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Small animal models of HIV infection are needed to investigate various fundamental and clinical aspects of HIV disease, i.e. host immune response, viral pathogenesis, and potential interventive therapies. Several classical and novel murine models are currently being studied for these purposes, but no one ideal model has been fully characterized. Within this report, we describe an additional murine model of HIV disease which uses sublethal gamma irradiated "nude" mice xenotransplanted HIV permissive CEM cells to serve as a nucleus of virus replication. Nude mice are transplanted with either 1X10⁷ HIV chronically infected or naive CEM cells and monitored for tumor progression and total body weight gain over time.

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The primary in vivo measure of HIV load is quantitation of a dynamic p24 gag antigenemia. Additional confirmation of virus replication is determined by immunohistochemistry for viral proteins, viral nucleic acids, TSEM, and recovery of infectious virus. This infectious HIV is recovered from mouse spleens 63 days after challenge, and is found without genotypic mixing with endogenous murine retrovirus or inhibition by endogenous host heat-labile serum factors. The nude mouse does not exhibit any signs or symptom of HIV infection, however follicular hyperplasia of the spleens of HIV bearing animals, along with non-heat labile virus neutralizing factors, indicate that these mice are capable of manifesting a immune response. Preliminary results indiate that oral administration of AZT reduces p24 levels in serum, and suggests that this model of HIV disease will have utility as a tool to assay in vivo candidate anti-viral compounds.

FOREWORD

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INTRODUCTION

I. NATURE OF PROBLEM

Much progress has been made toward the development of animal models of human immunodeficiency virus (HIV-1) disease. HIV-1 infection of nonhuman species (defined as when whole virus or viral protein products can repeatedly be detected and are usually associated with an immune response after a single challenge of virus[1]) has been attempted and is apparently successful in chimpanzees, gibbons and apes. It is generally agreed that these primates can be used only for an endpoint evaluation for the testing of efficacious vaccines, and not for the screening of therapeutic agents. Therefore, it is imperative that common laboratory animals such as the mouse be used as initial *in vivo* models for HIV experimentation.

II. BACKGROUND OF PREVIOUS WORK

Murine leukemia (MuLV) and feline immunodeficiency (FIV) virus models have been extensively studied[2,3] and are suitable to serve as a primary *in vivo* screen for many antiretroviral agents having potential therapeutic value in humans. The lentiviral genome of the HIV-1 is considerably more complex[4] than other known retroviruses, but therefore offers unique targets for specific anti-HIV-1 drugs[5]. This fact limits the predictive value of these model systems to agents that act on viral or cellular functions common to both HIV-1 and animal viruses. Because of this inherent problem, model systems which express infectious HIV-1 in human cells are desirable. In order to achieve this goal, some manipulation of the host and/or of transplanted cells is required. The resulting disadvantages of these models must be balanced against their expected utility.

The construction of transgenic mice containing HIV-1 proviral DNA provided the first approach to address this problem[7]. Pups of these transgenic dams displayed organomegaly, high degree of morbidity and mortality, and demonstrable

infectious virus. These animals appear to be most useful for studying the pathogenesis of HIV disease, but the small numbers bred limits their widespread utility.

Immune deficient mice have recently emerged as promising models of HIV-1 infection. Mice homozygous for the severe combined immunodeficiency (SCID) gene were characterized several years ago at the Fox Chase Cancer Center[8]. This mutant lacks functional T and B lymphocytes, but apparently possesses high levels of natural killer (NK) cells[9]. SCID mice have been reconstituted with either human fetal lymphoid tissue (SCID-hu mouse)[10], or human peripheral lymphocytes (PBL's) (hu-PBL-SCID)[11]. Using these approaches, these models have been successfully challenged with HIV-1, and this virus has been maintained and detected for varying periods of time[12,13]. Although promising in their utility to serve as a model of HIV spread within human tissues, and possibly for some pathogenesis studies, these SCID/HIV models are not without problems. First, the models require considerable expertise and manipulations to establish, perhaps being overly complex to assay antiviral agents *in vivo*, and viral titers are not consistently high, virus being detected often only by an overly sensitive polymerase chain reaction (PCR), which readily detects a single copy of viral DNA, but may be misleading with regards to interpretation complete viral replication. Second, the transplantation of human fetal tissue can involve ethical issues, which may limit the use of this approach at some institutions. Finally, the transplantation of PBL's from apparently healthy donors, can theoretically amount a graft versus host reaction against mouse tissue, and latently activated Epstein-Barr Virus[14] or cytomegalovirus can cause lymphomas or lymphoproliferation[15], or potentially complicate the interpretation of data from anti-viral drug testing.

III. PURPOSE OF PRESENT WORK

The "nude" or athymic mouse[16] offers an alternative approach for a small animal model of HIV infection. Although the nude mouse is deficient in functional T lymphocytes[17], it retains some immunological competence. These animals produce IgM (although at lower levels than thymus bearing mice) and have significant levels of lymphocyte activated killer (LAK) and NK cells[18]. NK cells are notably less reactive in immature and aged animals[19], and both LAK and NK cell activity can be reduced by total body irradiation[20] or specific antibodies directed against these cells[21]. Nude mice have been used extensively to assess the susceptibility of malignancies to chemotherapy, radiotherapy, immunotherapy, hormonal manipulation, or biological response modifiers, either alone or in combination[22]. Although less extensively utilized these animals have proven to be useful models for infectious agents which have been difficult to assay in other animal systems[23].

The acquisition of new virus associated antigens by tumor cells through infection with viruses and subsequent recognition of the new antigens as foreign by the tumor-bearing host is not a new concept[24]. The process of "viral xenogenization" has been suggested for use as a biological response modifier to control neoplastic growth[25]. In an attempt to test the effects of viral xenogenization on heterotransplanted tumors, Reid et al[26] transplanted mumps virus-infected human HeLa cells or vesicular stomatitis virus infected hamster BHK cells into nude mice. Tumors were produced, but at decreased size and incidence when infected with either virus, suggesting a host response to the neoantigen. More importantly, infectious viral particles were only found when they were given 500 total body rads of ^{137}Cesium irradiation. These results provide a firm foundation that human viral permissive cells can proliferate in athymic mice, and that RNA viruses capable of replicating in these cells can produce complete virus in hosts not normally associated as recipients. Although the natural history of the virus infection

is artificial, the virus production *in vivo* is not. Based upon these findings, we now describe an approach for the establishment of the xenotransplanted nude mouse as a model of HIV-1 infection. This model should have utility for therapeutic and limited immunological investigations.

IV. METHODS OF APPROACH

The overall approach used in the investigations studied under this contract was to determine if the immunosuppressed mouse xenotransplanted with a human HIV-permissive cells was capable of supporting HIV replication and to refine established techniques (laboratory tests), as applied to the model system, capable of assessing the replicative cycle of HIV.

Our approach consisted of an initial phase where the dynamics of uninfected CEM transplants were determined, and the effect of the tumor on the mouse was assessed. Specifically, tumor size and growth kinetics, animal morbidity (as assessed by the ability of the animal to thrive), and the role of gamma irradiation in tumor establishment were determined. After these baselines were established, we challenged tumor bearing mice with HIV-infected CEM cells. The effect of the virus on baselines was assessed and virus was detected both in tumor tissue (through nucleic acid analysis and immunohistochemistry) and in plasma (through p24 antigen assay). The next approach taken was that of determining if an initial inoculum consisting of both infected and non-infected cells (thereby providing uninfected target cells in the inoculum) would result in HIV replication in the mouse and if there would be any effect on the baselines established earlier. Various ratios of infected to non-infected cells were used, including 100% infected. Virus was detected as above.

Assessment of viral distribution in highly antigenemic mice through extensive histopathology, tissue p24 levels, virus neutralization and antisyncytial activity

assays, and plots of antigenemia from 3 days through nine weeks *in vivo* permitted determinations of viral load and some host immune responses, as well as presence of heat-labile mouse inhibitory factors. In other collaborations, attempts at the establishment of CEM transplants in neonatal BALB/c mice were made and the successful HIV challenge of HIV-permissive heterotransplants in immunosuppressed adult BALB/c mice accomplished (see midterm and appendix).

During the final stages of this USAMRDC contract, preliminary studies involving a potent antiretroviral agent, i.e. azidothymidine, have been initiated. This work involved the direct challenge of HIV into developing CEM transplants, and continues to be optimized with funding provided by the pharmaceutical industry.

BODY

I. EXPERIMENTAL METHODS

A. Cell Lines and HIV Cultures. CCRF-CEM or CEM is a well characterized[27] tumorigenic[28] and HIV permissive[29] cell line that was acquired from the American Type Culture Collection, Rockville, MD. and maintained in Roswell Park Memorial Institute (RPMI) 1640 medium with 15-20% fetal calf serum as described previously[28].

The CD4 + human lymphoblastoid cell lines CEMx174[30], MT-2 (obtained from D. Richman, Veterans Administration Medical Center, University of California, San Diego), and H9 (obtained from R.C. Gallo, National Cancer Institute), were cultured at 37^{0}C in RPMI-1640 containing 12% heat-inactivated fetal bovine serum and 50 ug of gentamicin per ml. Stocks of the HIV-1 isolate HTLV-IIIB (obtained from R.C. Gallo) were harvested from cultures of chronically-infected cells. Virus-containing culture fluids were clarified of cells by low speed centrifugation and passed through 0.45 micron filters. Infectious virions were quantitated by microtitration on MT-2 cells

using cytopathic effect (CPE) as end point for infection[31]; the reciprocal dilution at which 50% of wells showed CPE after 2 weeks incubation defined the infectious titer (1 TCID50). HIV-1 producing cells used for mouse transplantation were standardized to 10^6 infectious units/mL.

B. Mice and Cell/HIV Transplantation.

Athymic 3-4 week old (nu/nu) nude mice were purchased from Harlan Sprague Dawley, Inc. and were housed, maintained inoculated, and monitored daily for weight gain and CEM tumor progression as previously described[32,33]. All mice were exposed to 609 (+/-15) Roentgens at the body surface using a ^{137}Cesium source 24 hours prior to CEM cell transplantation. Additional biosafety precautions as recommended[34,35] were adhered to. In order to avoid the use of HIV-1 infected needles, a 22 gauge teflon angiocatheter was inserted at the inoculation site subcutaneously, and the syringe containing 0.2 ml of CEM/HIV-1 cell suspension in serum-free RPMI 1640 medium was luer locked onto the catheter prior to injection. The tumor volume was calculated from measurements in two dimensions using the formula for a prolate ellipsoid, $\pi/6$ LW2[36]. Significance of differences between groups was evaluated by the unpaired Student t-test. Probability values are specified in two tails. For p24 antigen assays, representative samples of tumor, spleen, and kidney tissue were removed at necropsy, rapidly frozen in liquid nitrogen and stored at -80^0 C for subsequent processing. The control mice were sacrificed prior to the point when the tumor appeared burden was stressful.

C. TSEM and Dot Blots

CEM transplant tissue for ultrastructural studies was removed at necropsy and prepared for thin section electron microscopy (TSEM) as previously reported[37], and examined in a Phillips 200 electron microscope. Cellular DNA and RNA were extracted separately as described previously[38] and dot blotted to a nitrocellulose

filter membrane by the method of Kafatos, et al.[39] The nucleic acids were probed with ^{32}P-labelled HIV-1 specific DNA (1.1Kb Bam HI- Sst I nef region of the pBH10R3 plasmid[33].

D. p24 enzyme immunoassay

The p24 enzyme immunoassay (EIA) is the unmodified procedure commercially available from Abbott Laboratories (Chicago,Il. USA) first described by Allain, et al.[40], and is utilized on tissue culture supernatants[41], heparinized mouse plasma, and mouse serum[33]. The neutralization of reactive mouse samples is achieved by the addition of human polyvalent serum supplied by Abbott Laboratories. The procedure described by Reka, et al.[42] with modifications, was used to determine the levels of p24 antigen within mouse tissues. Approximately 0.1 g of previously frozen tissue obtained at necropsy was weighed and added to 1.0 ml of phosphate buffered saline (pH 7.2) containing 1.0% triton X-100. The tissue was homogenized using a glass pestle in a 15ml glass homogenization tube on ice. The homogenates were centrifuged at 12000xg at 4^{0}C for 45 min to remove residual cellular debris. Supernatants were then assayed for p24 antigen using the procedure described. Total protein content was determined using a microlowry assay as described by Peterson[43] (Sigma Chemical Company, St.Louis, MO). Results were standardized to femtograms p24/milligram protein. The Mann-Whitney test was used to determine significance of difference in levels of p24 antigen between various tissues assayed.

E. Microscopic examination of Spleens

At necropsy, the spleens of control and HIV-1 challenged mice were removed and placed in 10% formalin for fixation. Tissues were paraffin embedded, sectioned, and stained with Hematoxylin and Eosin. Sections were scanned by light microscopy for hyperplastic follicles, and relative numbers of follicles were scored (negative to 3 +) per low power (100X) field.

F. Virus Neutralization

Virus neutralization was measured in 96-well microdilution plates as previously described[31]. Briefly, 2-fold serial dilutions of mouse plasmas were made in triplicate in a total of 100 ul growth medium. Fifty microliters of virus-containing medium were added to all wells except for 1 row of eight non-cytopathic control wells, which received growth medium in place of virus. MT-2 cells (10^5 cells in 100 ul of growth medium) were then added to each well. Viral-induced CPE was quantitated by vital dye (neutral red) uptake in remaining viable cells 3-4 days later. Percent protection is defined as the difference in light absorption (A540) between test wells (cells + test serum + virus) and virus control wells (cells + virus) divided by the difference in light absorption between cell control wells (cells only) and virus control wells. Plates were harvested when >80% CPE was present in the virus control wells. Neutralizing titers were defined by the reciprocal of the last dilution resulting in 50% or greater protection.

G. Antisyncytial Activity

Antisyncytial activity in mouse plasmas was measured by mixing MT-2 cells with H9/HIV-1 IIIB cells at a 10:1 ratio in the presence and absence of plasmas in 96-well microdilution plates, and measuring syncytium formation and CPE 18-24 hours later as described[44]. Serial dilutions of plasmas were made in triplicate. MT-2 cells ($1.5x10^5$) in 100 ul of growth medium were then added to each well. HIV-infected H9 cells ($1.5x10^4$) in 50 ul of growth medium were added to all wells except one row of 8 non-cytopathic control wells, which received uninfected H9 cells. Syncytium formation leads to, and is directly proportional to, cytopathic effect in this assay[44]. After incubation at 37^0C for 20 hours, syncytium formation was observed microscopically while viable cells were measured by vital dye uptake as described above.

H. Recovery of infectious virus and characterization of genotype

Three mice were transplanted with a mixture of 90% uninfected CEM cells/10% HIV-1 chronically infected CEM cells (total of $1X10^7$ cells) as described above. After 63 days the animals were sacrificed, p24 serum levels were determined, and spleens were aseptically removed. Each spleen was minced and added to 15ml of RPMI containing 20% fetal calf serum. After overnight incubation at 37^0C the spleen cells were co-cultured with CEMx174 cells for 12 days. After 12 days the majority of the cells were intensely positive for HIV-1 antigens as measured by IFA, and exhibited some CPE. Culture supernatants were then used to infect H9 cells in order to obtain chronically infected stock cultures. The majority of cells in these infected H9 cultures were IFA positive for HIV-1 antigens after 13 days. Infected H9 cells were solubilized in a solution of 10mM Tris-HCL pH 8.9, 10mM EDTA, 150mM NaCl, 0.5% SDS and 200 ug/ml proteinase K overnight at 37^0C[38]. Chromosomal DNA was extracted with phenol/chloroform and precipitated in ethanol[45]. DNA (10 ug) from each sample was digested with a restriction endonuclease (Sac I or Hind III), fractionated in a 0.8% agarose gel, denatured, and blotted onto a positively charged nylon membrane (GeneScreenPlus, DuPont, Wilmington, DE). Membranes were hybridized to either the 9.0 kb pBH10 probe[46] or 8.2 kb pMoMuLV probe[47]. Probe DNAs were labelled with ^{32}P by nick-translation. Hybridizations were performed at 42^0C overnight. Membranes were washed twice in 2X SSC at room temperature (RT), twice in 2X SSC/1.0% SDS at 65^0C, twice with 0.1X SSC at RT, and air dried prior to exposure to X-ray film.

I. Indirect immunofluorescence assays. HIV-1 antigen-expressing cells were detected by IFA using a 1:50 dilution of serum from a HIV-1 positive individual (confirmed positive by western blot), and a 1:200 dilution of fluorescein conjugated IgG fraction goat anti-human IgG. Slides were prepared by air drying and fixing in a

50:50 mixture of acetone/methanol for 30'. Slides were counterstained with Evan's blue[48].

J. AZT administration. Animals were started on a dose of AZT (dissolved in drinking water) one day prior to viral challenge (R. Schinazi, personal communication). All mice were followed for three or four weeks, and sacrificed by exsanguination for determination of p24 serum levels. At the time of sacrifice, a large representative sample of tumor was aseptically removed, and placed into a tissue culture flask with 10ml RPMI 1640 medium/10% serum. These cultures were incubated for 4hrs. at 37^{0}C the permit the dissociation of the mass into individual cells. The resulting tissue cultures were processed for qualitative IFA and semi-quantitative TCID50 assays. These assays were blinded as to drug treatments and p24 gag serum levels.

II. RESULTS OBTAINED

Our initial experiments were designed to determine if the CCRF-CEM (CEM) cell line could be transplanted into gamma-irradiated nude mice and provide predictable tumor incidence and progression with minimal morbidity from irradiation or tumor burden. Various cell inocula ($0.5X10^7$; $1.0X10^7$; $2.0X10^7$; and $5.0X10^7$ cells) were injected into irradiated and non-irradiated mice. Body weights and tumor volumes were recorded five times a week for thirty-five days. Tumors were detectable in all groups twelve to fourteen days after inoculation. The exponential phase of tumor development was delayed by approximately five days in all non- irradiated groups. Regression of individual tumors occurred in 16 out of 20 non-irradiated animals. Weight gain was similar for all groups. Figure 1 shows the effects of irradiation on weight gain and tumor growth in mice injected with $1x10^7$ cells, the inoculum which produced consistent tumor growth without necrosis.

Presence of the major core p24 gag protein of the HIV-1, p24 gag, circulating in the bloodstream, as detected by antigen capture assays, has significant clinical implications. Variations in the plasma antigen levels of HIV-1 infected individuals is accepted as an important indicator of disease progression in both hemophiliac[40] and homosexual[49] populations. In addition, a suppression in plasma levels of p24 antigen is associated with a positive therapeutic response to candidate antiviral agents[50]. Therefore, we determined the presence of p24 antigen plasma by EIA with antibodies raised in rabbits.

In order to establish optimum conditions for HIV-1 replication in CEM xenotransplants in nude mice, four populations of gamma-irradiated four week old female nude mice were injected with suspensions of $1X10^7$ CEM cells containing varied amounts (0, 10, 50, or 100% of total cell population) of HIV-1 infected

Cells. Cell mixtures were loaded into individual syringes and injected along with a total inoculum volume of 0.2 mL serum-free RPMI. To explore the possibility that an established heterotransplant of CD4+ target cells may be necessary for successful HIV infection, an additional population of mice, previously inoculated seven days earlier with $1X10^7$ uninfected cells, with small and progressing lymphomas (mean size) received an injection directly into the tumor mass with $0.5X10^7$ infected cells from a CEM/HIV-1 culture. Infectivity of HTLV-III_B/CCRF- CEM cell culture was confirmed by indirect immunofluorescence of surface antigens. Antigen production of the infected culture was determined to be 1550 pg/mL. All animals were sacrificed after 23 days and 200 ul of plasma were used to determine p24 antigen levels. Inocula used and results are given in Table 1.

Table 1: p24 Antigenemia in athymic "nude" mice challenged with a mixture of HIV-1 chronically infected and naive CCRF-CEM cells

Number of CCRF-CEM Cells Inoculated	Per Cent of Inoculum With HIV	Population Size Positive Animals	Number of p24 Antigen Positive Animals	Average Plasma Antigen of pg/ml (SEM*)
$1X10^7$	0	5	0	0
$1X10^7$	10	6	6	130 (8%)
$1X10^7$	50	6	5	70 (21%)
$1X10^7$	100	6	3	15 (8%)
$1X10^7$	0	6	1	9
$+0.5X10^7$**	100			

* Standard Error of the Mean
** Challenge after palpable tumor detected

The 10% infected group had the most consistent result with all animals positive for HIV-1 antigen. Incubation of these samples with human HIV-1 neutralizing antibodies (Abbott Laboratories) reduced the antigen value of each sample to background levels. In control mice (injected with CEM cells, but not challenged with HIV-1), plasma assay values were below background human plasma readings; this differs from the high background produced by undiluted rabbit serum[51] in this assay.

HIV-1 infection had no significant effect on weight gain in the mice. Significant differences in tumor volumes occurred in the 10% infected inoculum group, with the average tumor volume 50% less than the average of the control group on day 23 after inoculation. Electron microscopic examination of tumor sections showed rare lentiviral particles within intracellular vesicles (Figure 2A). Dot blots of nucleic acids from the same tumor tissue probed with ^{32}P-dCTP labelled HIV-I specific DNA, 1.1 Kb BamHI-SstI *nef* region detected viral RNA in 5 micrograms of total cellular RNA

(Figure 2B). Plasma level of p24 antigen from this same mouse was determined to be 213 pg/ml. After neutralization with human HIV-1 antibodies, plasma was assayed again for p24 antigen and was below detectable limits.

To determine the clearance of p24 antigen *in vivo*, four gamma- irradiated mice were injected intraperitoneally (i.p.) with 0.5 ml of cell-free tissue culture supernatant from a HIV-1 infected CEM cell culture producing 500,000 pg/mL p24 antigen. A control mouse was injected i.p. with uninfected CEM cell culture supernatant. Virus challenged mice were sacrificed at 24 hours, 48 hours, 7 days, and 14 days. Plasma from the control mouse was non-reactive for p24 antigen at 48 hours. Of the virus challenged mice, only in the mouse sacrificed at 24 hours was antigen detectable (2.9 pg/mL). All other plasma results were below detectable limits. These findings suggest that in the absence of human target cells, exogenously supplied viral proteins are quickly metabolized by the mouse.

We determined the course of antigen production over time in HIV-1 infected nude mice. Four week old gamma-irradiated male nude mice were inoculated with $1x10^7$ CEM cells, 10% of which were chronically infected with HIV-1, or uninfected (control). The animals were monitored daily for 63 days, and at each of eight test points, one group of 6 mice was sacrificed, plasma collected, spleen removed for histological examination, and tissues frozen for p24 antigen levels. At each test point, one group of 6 mice was sacrificed and plasma p24 antigen levels determined. Figure 3 shows the average antigen level of the group at each time point. Antigen was detectable at 3 days after inoculation (28 pg/ml), spiked at 5 days (514 pg/ml), rose steadily from 7 to 42 days (90-2181 pg/ml), then dropped off at day 63 (1269 pg/ml). It is not clear at this time what is responsible for this dynamic antigenemic state, but it could be due to either the mouse immune system[52], the viral replicative cycle[53], or CD4 + receptor regulation by the CEM cells[54], however the antigen curve indicates a strong similarity to *in vitro* HIV-1 replication[55].

Average tumor volumes for each group and for the control mice are also shown in Figure 3. Normal tumor progression with exponential growth is evident in the control group. Among the infected mice, tumor volumes were significantly less ($p<.05$) than controls. Between 6 and 9 weeks, the tumors of the remaining infected mice entered an exponential growth phase. The plasma antigen levels in these mice were found to be much lower than in the previous groups, indicating that the virus has an inhibitory effect on tumor development. These findings are in agreement with the reported antitumorigenic effects of infectious RNA (mumps) virus replication in HeLa cells xenotransplanted into gamma-irradiated nude mice[26].

In order to determine the tissue sites of HIV-1 p24 antigen production, mouse spleens and kidneys, and CEM tumor tissue from the animals described above were processed for the presence of this protein. Figure 4 displays these results, as well as plasma levels adjusted to fg p24/mg total protein. Measurable levels of p24 was found in all tissues sampled during the nine week course of the experiment, with significant differences ($p = .02$-$.03$) occurring between both mouse tissues and plasma at 9 weeks. No significant differences occurred earlier, except for a difference ($p = .02$) between spleen and plasma levels at week three. The tumor p24 levels clearly indicate that this is the primary site of viral replication, but other sources of viral proteins exist, indicating that a host immunological response could manifest due to the relatively high levels of p24 within these spleens.

No mortality or disease symptoms from HIV-1 infection were observed in any of these mice. Splenomegaly was seen in 5 of 12 infected animals starting at forty-two days after infection; by day sixty-three, the spleens of all the remaining infected animals displayed readily apparent white pulp. Microscopic examination of spleen sections revealed reactive follicular centers surrounded by a hyperplastic zone, suggestive of a host B-cell response (Figure 5). This clonal B-cell activation is reminiscent of the hyperplastic B cell response found in early AIDS[56,57], and

suggestive of a likewise similar hypergammaglobulinemia also found in these patients[58]. Histological sections of the spleen reacted with rabbit or sheep antiserum against p24 core protein, visualized by the peroxidase reaction (see midterm report) indicated reactivity with histiocytes and dendritic reticulum cells (midterm report), both cell types which have important roles in HIV pathogenesis[59]. Because of these findings, we anticipated the production of a detectable humoral response.

The major scientific reason for the lack of continued USAMRDC support for the HIV-1 xenotransplanted nude mouse *in vivo* system was the discovery by Hosoi et al[60] of a "potent heat-labile antiviral effect shown in the serum of mice which will not be able to give reliable data regarding the effect of drugs on infectious cell-free virus, as cell-free virus is rapidly inactivated by the serum factors". Based upon this discovery, we attempted to confirm the presence of this factor and determine the relevance of this finding to our model system, and compare it to a non-heat-lable (i.e. antibody) host response.

Plasma from HIV-1 naive mice were found to possess an unidentified, heat-labile component capable of neutralizing cell-free virions and blocking HIV-1 induced syncytium formation. We found this activity in 3/3 Swiss outbred mice and in 5/5 nu/nu mice when fresh-frozen plasma were used (Figure 6) but not when plasma were heat-inactivated for 1 hour at 56^{0}C (data not shown). Heat-labile neutralizing titers (reciprocal of last dilution giving 50% or greater protection) ranged from 100-500 in the nu/nu mice, while heat-labile antisyncytial titers were considerably lower. Heat-labile neutralizing activities in plasmas from nu/nu mice were relatively similar while those from Swiss outbred mice were disparate. In fact, plasma from one Swiss outbred mouse had biphasic neutralizing activity, where HIV-1 was neutralized at dilutions of 1:20 and 1:100 - 1:1000 but not at dilutions between 1:20 and 1:100. The presence of uninfected CEM xenotransplants had no

obvious effect on the heat-labile neutralizing or antisyncytial activities of plasmas from nu/nu mice.

In contrast to heat-labile neutralizing and antisyncytial results, plasmas from 7/7 nu/nu mice harboring infected CEM xenotransplants had heat-stable, HIV-1 neutralizing titers of 1:72 to 1:288 (Table 2). This neutralizing activity appeared by day 7 post inoculation (mouse 1) and was retained in plasmas even after two cycles of heat-inactivation.

TABLE 2: HIV-1 IIIB neutralizing antibodies in plasmas from nu/nu mice harboring infected CEM xenotransplants.

	Neutralizing titers (reciprocal dilution)		
Mouse	day 7[1]	day 14	day 21
1	288	288	288
2	NT[2]	NT	72
3	NT	NT	288
4		NT	144
5	NT	NT	144

[1] Days post inoculation with cells.
[2] NT, not tested.

Next we wished to determine whether infectious HIV-1 could be recovered from previously challenged mice. Three mice were transplanted with the infected with HIV-1 (see methods) were harvested at 63 post challenge, and the initial % IFA positivity of the tumor transplants for mouse A1, A3, and A4, were 80, 60, and 100 respectively, and the p24 serum antigen and tumor volumes at sacrifice are displayed in Table 3.

Table 3.Tumor volumes and p24 levels of mice with HIV-1 isolated from their spleens

MOUSE (A) (spleen cult)	TRANSPLANT VOLUME (CC^3)	SERUM p24 ANTIGEN (pg/ml)
1	31.9	604
3	24.7	525
4	7.0	627

The DNA blots from cells of the spleen cultures are displayed in Figures 7 and 8. These blots indicate that HIV-1 can be recovered from a mouse organ distal from the primary source of infection, without evidence of genotypic mixing, deletions, or rearrangements. While these experiments do not address the potential concerns regarding phenotypic mixing and expanded virus trophism[61,62,63], these results indicate, along with our findings on serum inhibitory factors, that this proposed animal model of HIV infection should still be useful for antiviral studies.

Azidothymidine (AZT) is the current gold standard for investigations involving anti-HIV agents, and has successfully been used as an anti-HIV agent in the SCID-hu mouse model[64,65]. To investigate whether AZT has any effect upon serum p24 levels, we administered varying doses of AZT prophylactically (.25 to 2.0 mg/day/mouse p.o.) to groups of mice bearing human CEM cell transplants. The animals were challenged with 10^5 TCID50 units of HIV by injection of the 0.1 ml inoculum directly into the palpable mass. After 3-4 weeks of treatment the mice were sacrificed. Serum p24 levels and excised CEM cell HIV antigen expression (by IFA) were measured. The levels of these HIV indicators were significantly reduced in the AZT treated animals (Tables 4 & 5). Since anti-neoplastic drugs can affect the course of other murine models of AIDS[66], and Since AZT was originally designed as a cancer drug, we wished to assay for other biological AZT effects in the nude mouse model, levels of AZT as high as 8 mg/day/mouse did not exhibit any mortality,

morbidity, or antineoplastic effects over a 35 day period, (Figure 9) suggesting that the inhibition of HIV demonstrated earlier is due directly to the action of AZT on the HIV. Our early results indicate that this model of HIV disease can potentially be used to evaluate anti-HIV agents *in vivo*.

Table 4. HIV-1 challenge of progressively growing CEM transplants

MOUSE	% IFA POSITIVE	TCID50/ML	p24 (pg/ml)
A1	NT	NT	NT
A2	50	3.9 x 105	63
A3	1	<125	<3
A4	20	3.1 x 103	>600
A5	5	NT	<3
B1	1	625	8
B2	<0.1	NT	<3
B3	<1	<125	<3
B4	NT	NT	<3
B5	0	<125	<3
C1	NT	NT	<3
C2	NT	NT	<3
C3	0	NT	<3
C4	0	<125	<3
C5	<1	125	73
D1	<0.1	<125	<3
D2	NT	NT	<3
D3	0	<125	<3
D4	NT	NT	<3
D5	0	<125	<3
E1	NT	NT	<3
E2	NT	NT	<3
E3	<0.1	<125	<3
E4	<1	<125	<3
E5	0	NT	<3

A = Control no AZT
B = 0.25 mg/day P.O. AZT
C = 0.5 mg/day P.O. AZT
D = 1.0 mg/day P.O. AZT
E = 2.0 mg/day P.O. AZT
NT = Not Tested

Mice were injected directly into the palpable mass with 10^5 TCID50 units of HIV and followed for four weeks, while receiving one of four prophylactic doses of AZT in the drinking water. Transplants (i.e. tumors) were harvested, and assays for viral replication were determined. See Experimental Methods for specifics.

Table 5. HIV-1 challenge of progressively growing CEM transplants

MOUSE	Transplant Volume (cc3)	% IFA Positive Cells	p24 Antigen (pg/ml)
Placebo 1	4.7	50	2932
Placebo 2	19.5	10	11520
Placebo 3	14.0	<1	4912
Placebo 4	15.4	80	21888
AZT 1	8.5	2	<12
AZT2	16.0	20	918
AZT3	13.0	0	50
AZT4	22.1	0	<12
AZT5	9.7	0	243
AZT6	19.8	0	<12
AZT7	6.0	30	106
AZT8	6.7	<1	274

Mice were injected directly into the palpable mass with 10^5 TCID50 units of HIV and followed for three weeks with prophylactic AZT (2.0 mg/day/mouse) or water (control). Serum p24 levels and IFA antigen of tumor cells were determined. A 50 fold reduction of mean p24 levels is observed in mice with AZT treatment when compared to the control group.

III. COMPARISON TO GOALS

Based upon the milestones delineated in the workscope of the contract, we believe that we have met the goals set out in the original proposal. The timetable has been altered, due to setbacks in renovation, problems with technical support personnel, and most notably by a insidious murine hepatitis outbreak within our previously well contained specific pathogen free mouse facility. This infection seriously impaired our progress, and caused the loss of six to eight months of experimentation. However, as described below in the conclusion section, we believe that the pathogenesis of our model system has been well characterized and the utilities and limitations of this model have been reasonably understood, but require further investigations.

CONCLUSIONS

1. The nude mouse xenotransplanted with human CEM cells and HIV offers an approach towards a small animal model of *HIV infection* where most accepted parameters of viral replication can be measured.

2. The nude mouse does not exhibit signs or symptoms of HIV disease, but histological and immunological evidence suggests a specific host response to the viral infection.

3. Infectious HIV can be recovered from mouse tissue without evidence of genotypic mixing.

4. The trends of the IFA and TCID50 results reflect the inhibitory effects of oral AZT administration, and should correlate well, with *in vitro* findings.

5. AZT does not inhibit the growth of CEM xenotransplants. This finding removes an important parameter which could confuse the interpretation of anti-viral activity. Other candidate agents assayed in this *in vivo* system should also be controlled for anti-neoplastic activity.

6. Direct inoculation of HIV into the tumor does not appear to be the optimum route of viral challenge for *in vivo* anti-viral assays. Other approaches for virus challenge are currently being investigated.

7. The ease of separating CEM tumors into single cell suspensions has now enabled us to perform serial biopsies on the developing mass. This technique offers the potential to use flow cytometry or other automation to quantitate the time course of viral infection within the CEM transplants.

I. IMPLICATIONS

In summary, the results reported here demonstrate a system for the infection of nude mice with HIV-1 which produces a dynamic measurable antigenemic and viremic state. These clinically relevant parameters provide the means to investigate the intervention of HIV infection *in vivo* with candidate drugs. We anticipate this model system will prove useful to assay and study candidate anti-HIV therapies as well as offer insight into other aspects of HIV infection, including passive immunotherapy and host growth factor, cytokine, immune response, and HIV strain or isolate interaction.

II. FUTURE WORK

This work has continued to be supported by the DuPont Merck Pharmaceutical Company, to specifically provide an inexpensive mouse model capable of supporting the replication of HIV-1 to assay new candidate antivirals. It is anticipated that additional investigators and pharmaceutical companies will also find utility in this model system.

III. IMPACT ON PROBLEM TOPIC

Three and one half years ago, when this contract was first awarded, it was still not clear whether or where this model system, or similar murine models would have a role in investigating HIV disease. The major contribution provided by this research,

was not in specifically using the HIV, but rather in using a xenografted immunosuppressed mouse to establish a unique means of *in vivo* virus proliferation, in lieu of specific new host viral adaptation. Using this same xenograft transplantation principle, other investigators have now also established a systematic approach to study human B-lymphoma pathogenesis in SCID mice[67], as well as confirming the application of this principle in antiviral *in vivo* assessments[68,69]. While our foundation and approach toward this study has had some known, as well as previously unknown inherent limitations (as do all animal models), we anticipate that virus infected human cell lines xenotransplanted into immunosuppressed mice will continue to have acceptance and utility for basic science investigations, as well the rapidly expanding antiviral pharmaceutical industry.

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FIGURE LEGENDS

Figure 1. Weights and tumor volumes of athymic "nude" mice xenotransplanted with the human T-cell lymphoma cell line, CCRF-CEM. Each point represents the mean of five animals. Tumor volumes of the non-irradiated mice were significantly less than those of the irradiated mice (two-tailed Student's T test for unpaired data, $p<.10$). Irradiated animals: weights and tumor volumes (closed symbols). Non-irradiated animals: weights and tumor volumes (open symbols).

Figure 2A. Transmission electron micrograph of HIV-1 in transplanted CCRF-CEM cells. $1X10^7$ CCRF-CEM cells (10% of which were from a chronically infected HTLV-III_B/CCRF-CEM culture) were inoculated into a gamma-irradiated four week old nude mouse. Tumor tissue and plasma were collected at sacrifice, 23 days post inoculation. Plasma p24 antigen level in this mouse was determined at 213 pg/ml. Micrograph (X 79,750) shows two cells, one containing three virus particles in an intracellular vesicle (arrow). The typical bar-shaped nucleoid of lentiviruses is visible

Figure 2B. Dot blot of nucleic acids extracted from the same tumor tissue and from the infected cell line used to inoculate the mouse was probed with HIV-specific cDNA. Viral message and provirus were readily detected in 5 micrograms of cellular RNA and DNA, respectively, but only viral message was detected in the same amount of tumor-extracted nucleic acids. Uninfected cell line and spleen from a control mouse were both negative.

Figure 3. Plasma p24 antigen levels and tumor volumes of HIV-1 infected athymic "nude" mice. At indicated time points one population ($n = 6$) of mice was sacrificed and plasma tested for p24 antigen and tumor volumes calculated. The graph shows average antigen level (●) and tumor volume (o) for each population. Tumor volume

average for the control population (■) is shown for comparison. At 63 days after inoculation, tumor volumes of the infected mice were 50% less than those of control mice. (Reproduced in part from reference No.33, with permission.)

Figure 4. Distribution of tissue p24 levels. Tissue samples collected from the mice described in figure 3 were processed as detailed within the text, and the results were standardized to femtograms p24/milligram protein. The graph shows the average standardized antigen levels for plasma vs. three other tissues.

Figure 5. Distribution of mouse spleens exhibiting hyperplastic follicles. Spleens were collected from the mice described in figure 3 and fixed and processed and evaluated as detailed within the text. The graph shows the relative quantity of hyperplastic follicles viewed per 100x field for each individual mouse spleen in control (●) and challenged (♦) mice.

Figure 6. HIV-1 IIIB neutralizing and antisyncytial activity of plasmas from HIV-1 naive mice. Fresh-frozen plasmas (i.e., non-heat inactivated) were examined at dilutions of 1:16 to 1:2048 for neutralizing (closed symbols) and antisyncytial (open symbols) activities, respectively. A. Swiss outbred mice; B. Nu/Nu mice without CEM xenotransplants; C. Nu/Nu mice with 2 week-old, uninfected CEM xenotransplants.

Figure 7. Southern blot analysis of DNA extracted from murine (mu) tissue, spleen cell co-cultures, and HIV-1 positive control cells (H9/HIV), and negative control cells (H9). Spleens were obtained from 3 mice challenged with HIV-1 63 days earlier. Spleen cells were co-cultured with HIV-1 permissive cells as described in "Materials and Methods". The 9.0 kb pBH10 insert was used as a probe.

Figure 8. Southern blot analysis of the same DNA described in figure 7 legend, and hybridized with the 8.2 kb insert from the pMoMuLV.

Figure 9. Lack of anti-neoplastic effects on progressing CEM tumors. Four groups of 5 mice were inoculated with $1x10^7$ uninfected CEM cells and allowed to drink water (control) or a dose of AZT ad lib. Tumor volumes and body weights were measured daily for 35 days. The animals consistently drank 4ml/day/mouse and no morbidity or mortality was observed. Tumor volumes (shown above) and body weights did not demonstrate any significance difference using the two-tailed Student t- test for unpaired data.

Figure 10. IFA of CEM cells removed at necropsy, and dissociated from portions of CEM tumor tissue sample from AZT treated (1.0 mg/day P.O.) mouse D5 (right), or control (placebo) mouse (left). The cells and cultures were processed as described in experimental methods.

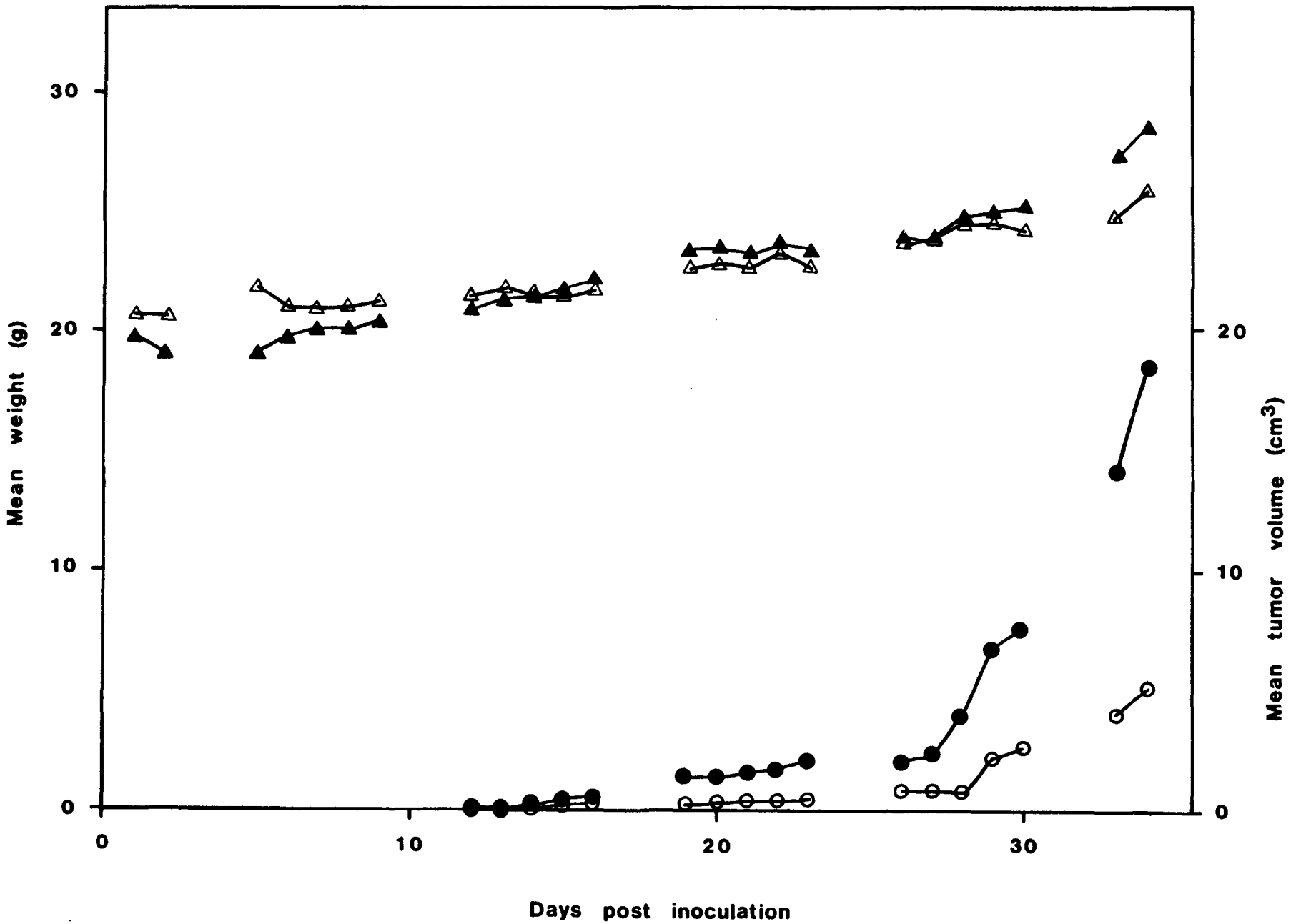

Mean weight (g)
Mean tumor volume (cm3)
Days post inoculation
0
10
20
30

figure 1

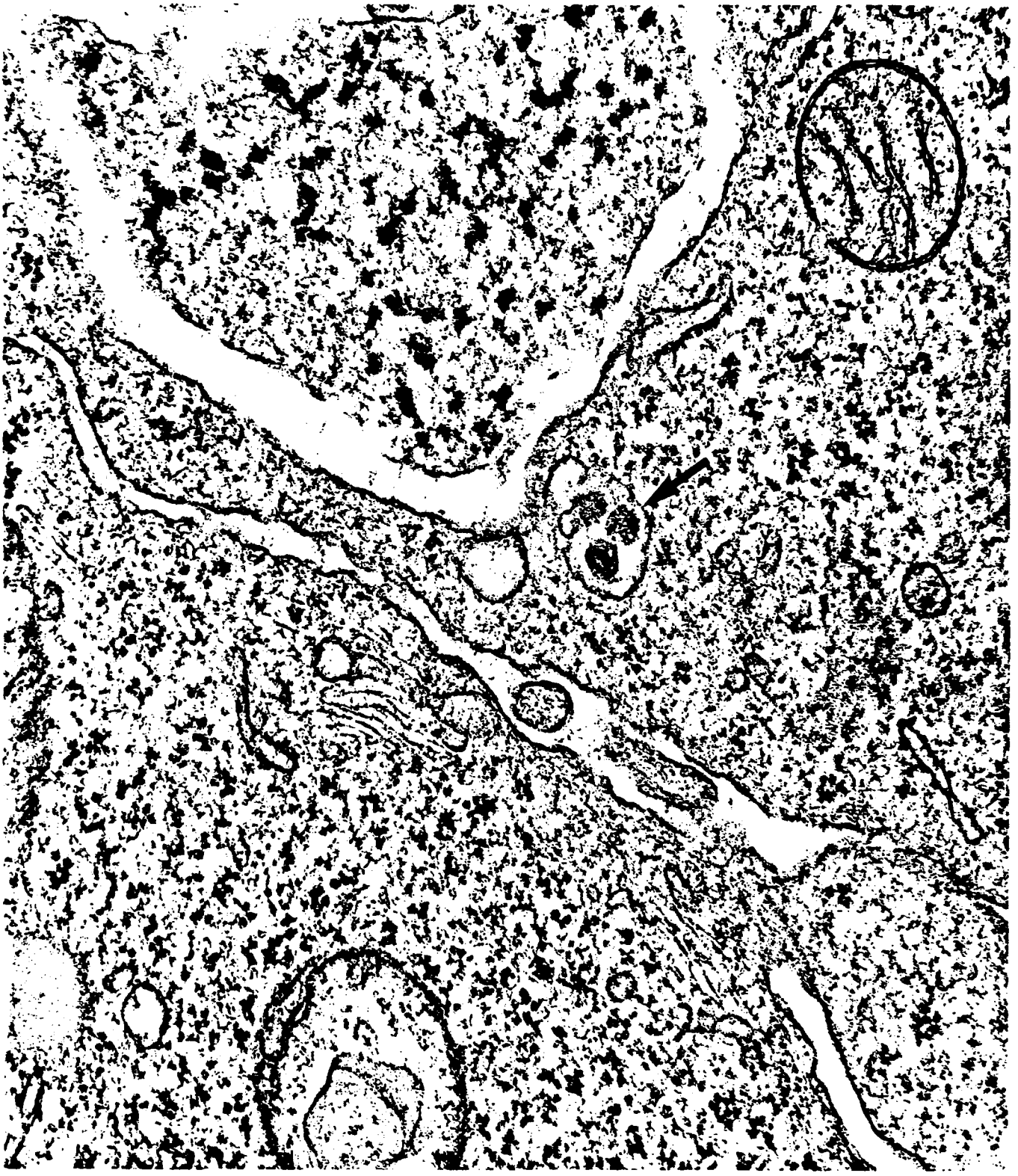


figure 2a

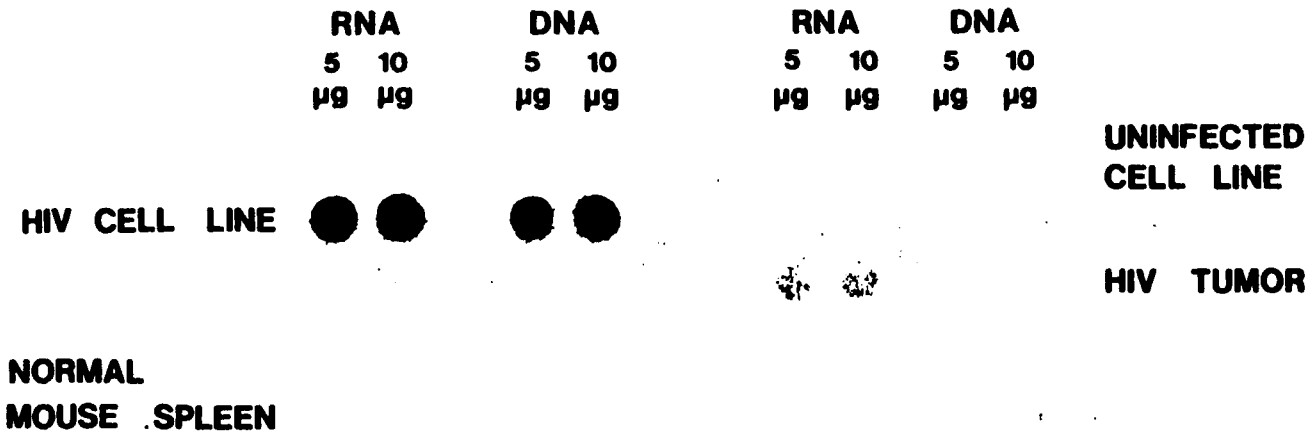

RNA
DNA
RNA
DNA
5 10
μg μg
5 10
μg μg
5 10
μg μg
5 10
μg μg
UNINFECTED
CELL LINE
HIV CELL LINE
HIV TUMOR
NORMAL
MOUSE SPLEEN

figure 2b

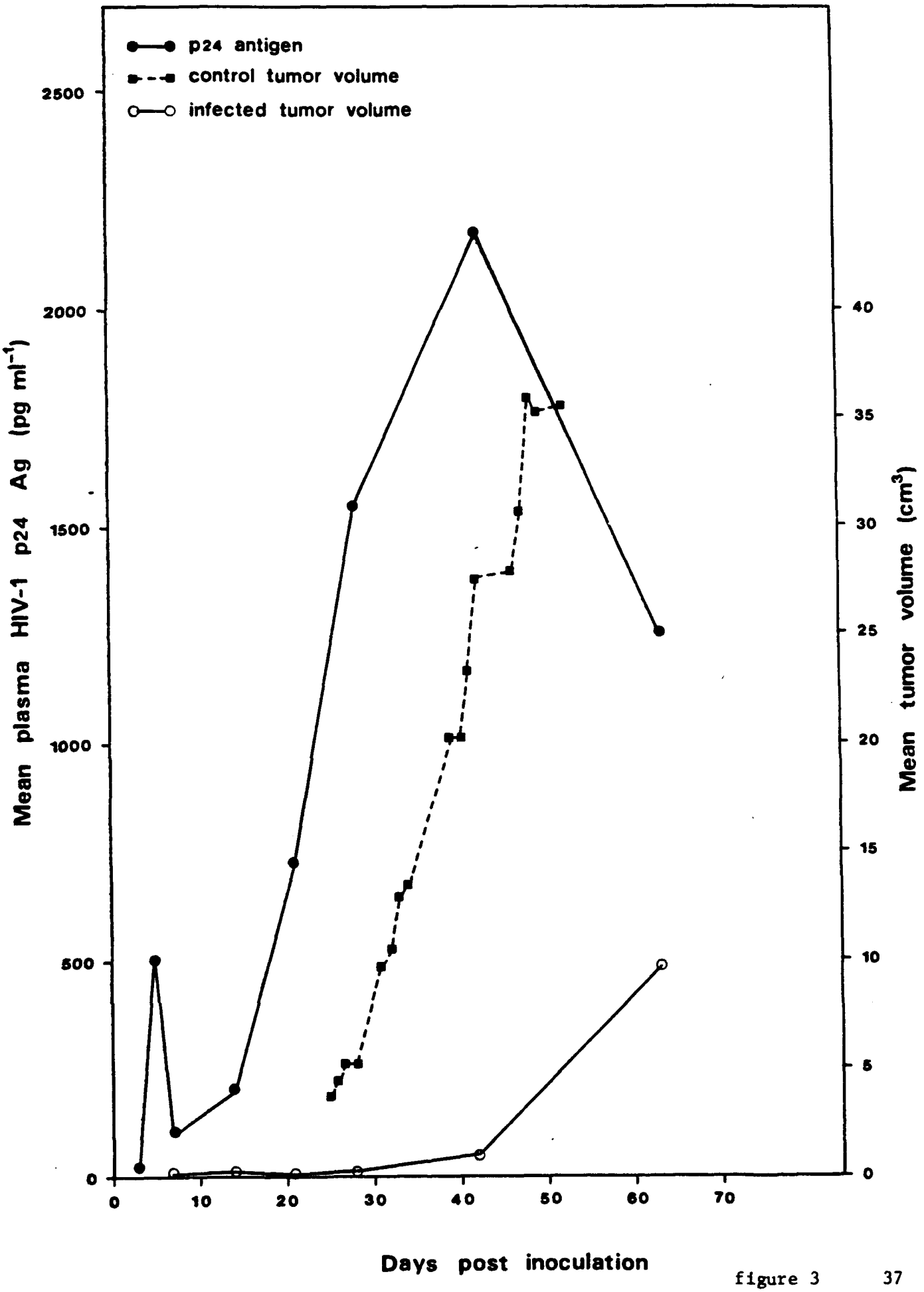

p24 antigen
control tumor volume
infected tumor volume
Mean plasma HIV-1 p24 Ag (pg ml^{-1})
Mean tumor volume (cm^3)
Days post inoculation
0
500
1000
1500
2000
2500
0
5
10
15
20
25
30
35
40
0
10
20
30
40
50
60
70

figure 3

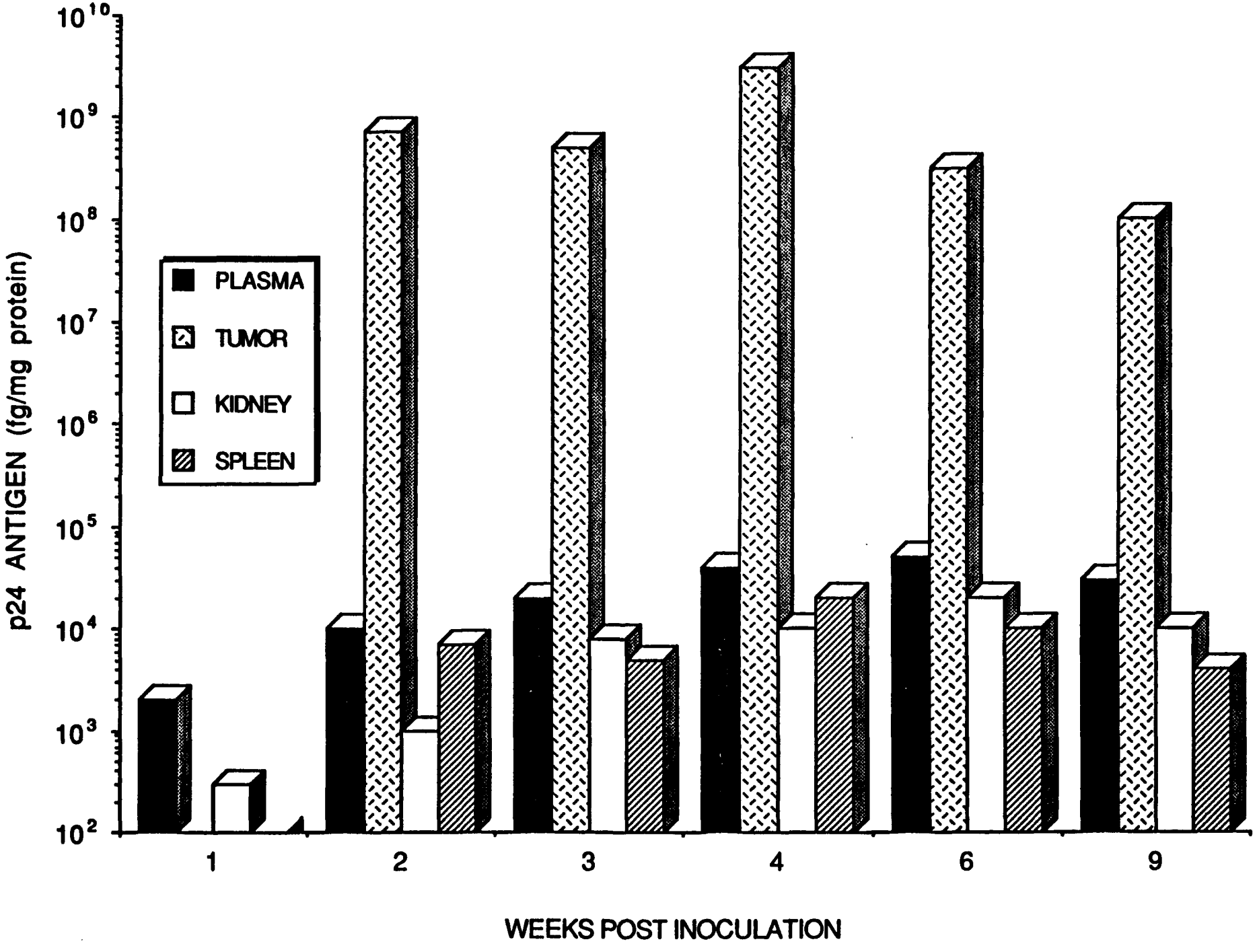

p24 ANTIGEN (fg/mg protein)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
PLASMA
TUMOR
KIDNEY
SPLEEN
1
2
3
4
6
9
WEEKS POST INOCULATION

figure 4

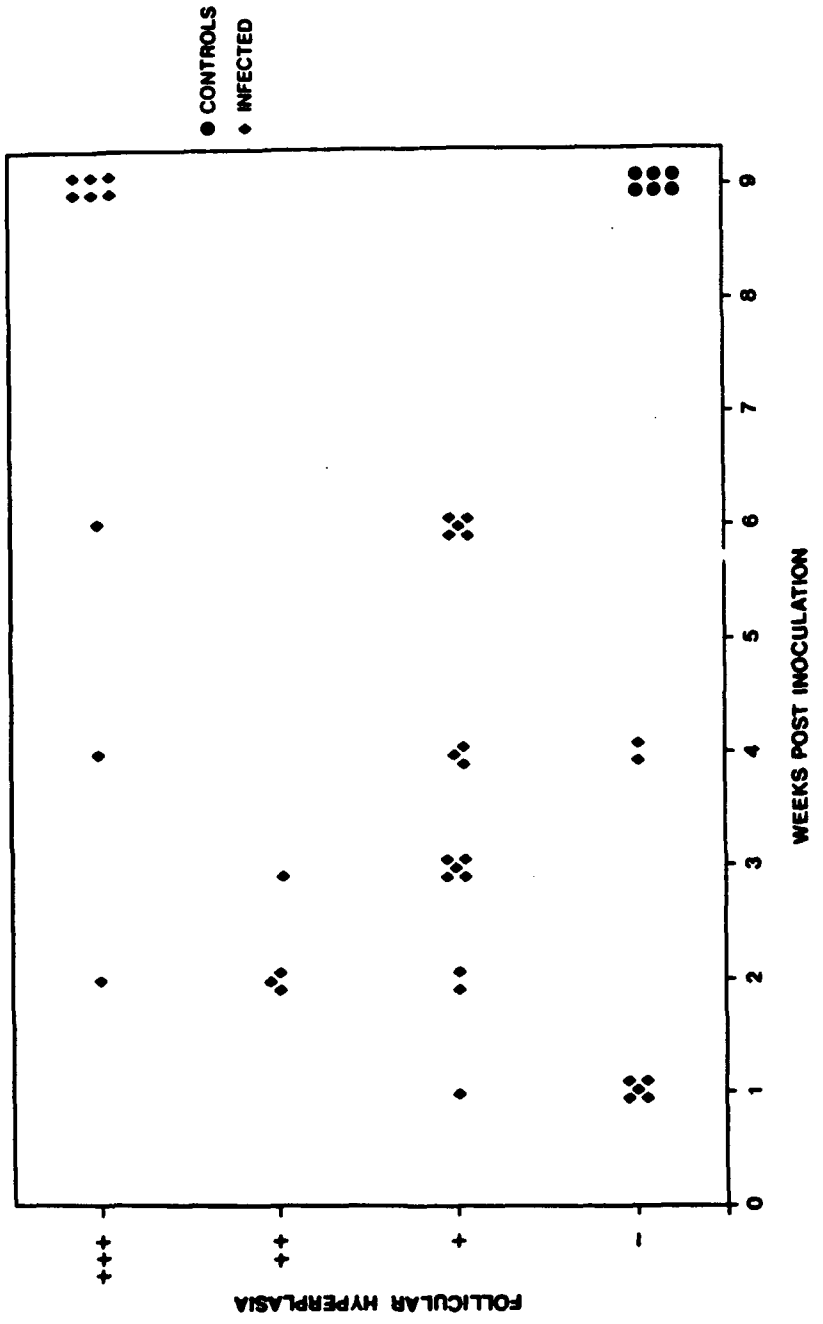

● CONTROLS
◆ INFECTED
FOLLICULAR HYPERPLASIA
+++
++
+
-
0
1
2
3
4
5
6
7
8
9
WEEKS POST INOCULATION

figure 5

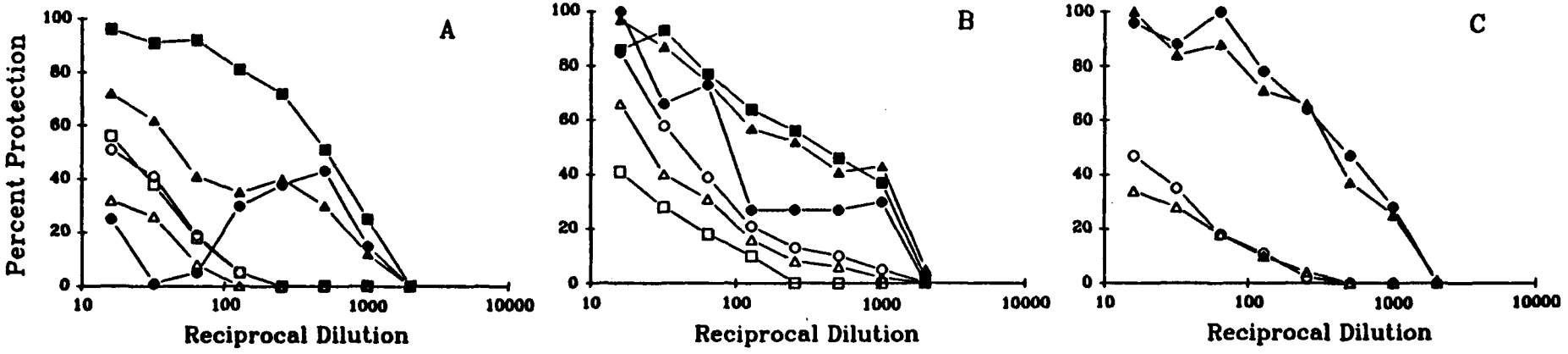
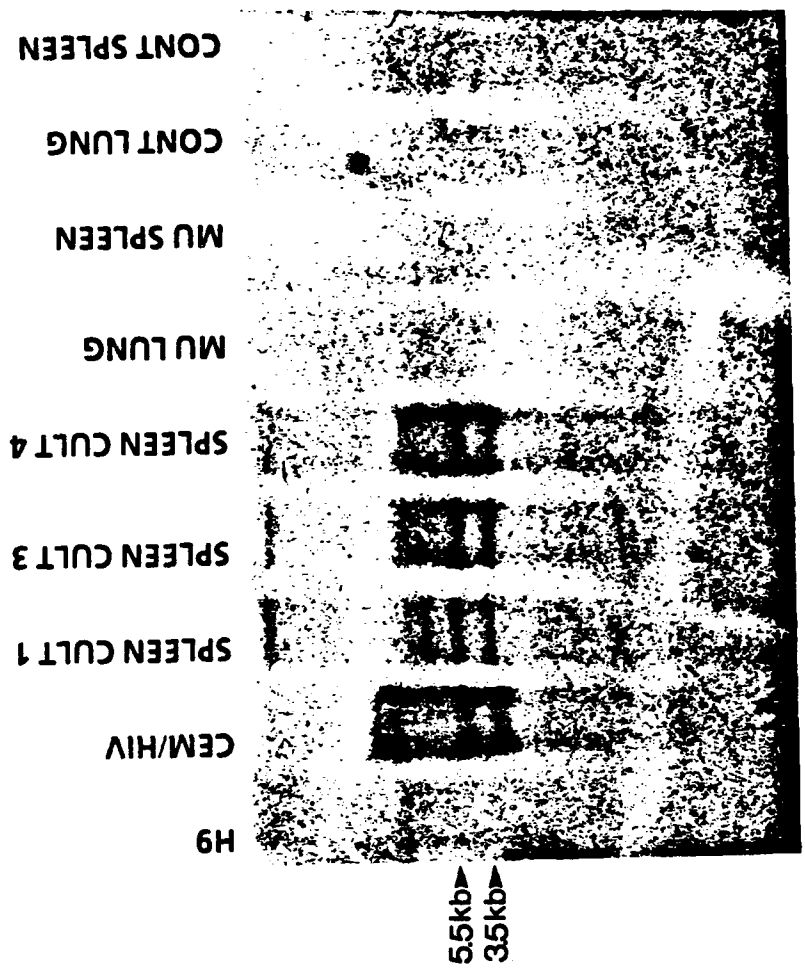

A
B
C
Percent Protection
Reciprocal Dilution
Reciprocal Dilution
Reciprocal Dilution
100
80
60
40
20
0
10
100
1000
10000

figure 6

figure 7


H9
CEM/HIV
SPLEEN CULT 1
SPLEEN CULT 3
SPLEEN CULT 4
MU LUNG
MU SPLEEN
CONT LUNG
CONT SPLEEN
5.5kb
3.5kb

8.2kb
PBH10
pMLV
Mu SPLEEN
Mu LIVER
Mu LUNG
SPLEEN CULT4
SPLEEN CULT3
SPLEEN CULT1
H9
H9/HIV

figure 8

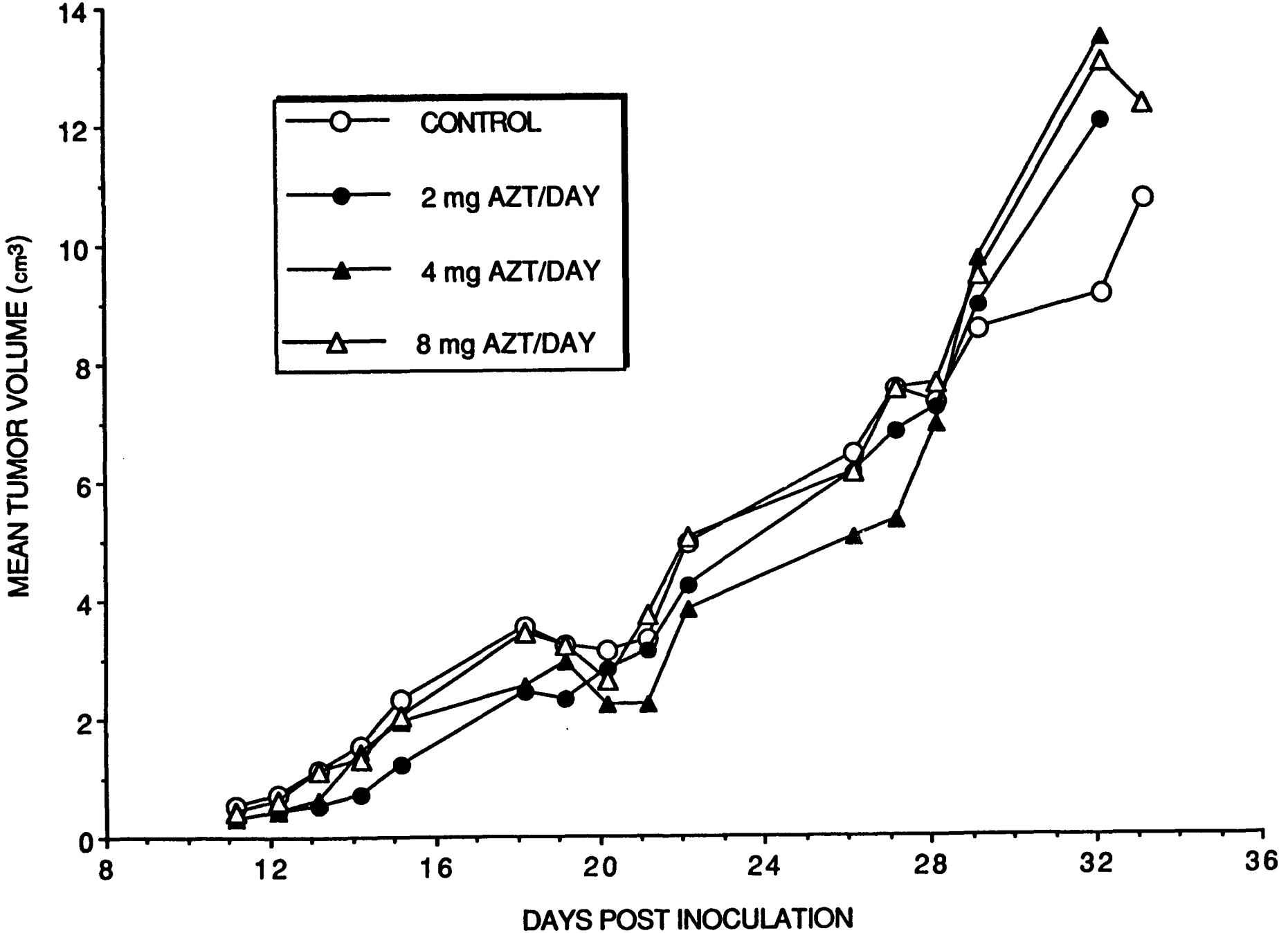

CONTROL
2 mg AZT/DAY
4 mg AZT/DAY
8 mg AZT/DAY
MEAN TUMOR VOLUME (cm3)
DAYS POST INOCULATION
0
2
4
6
8
10
12
14
8
12
16
20
24
28
32
36

figure 9

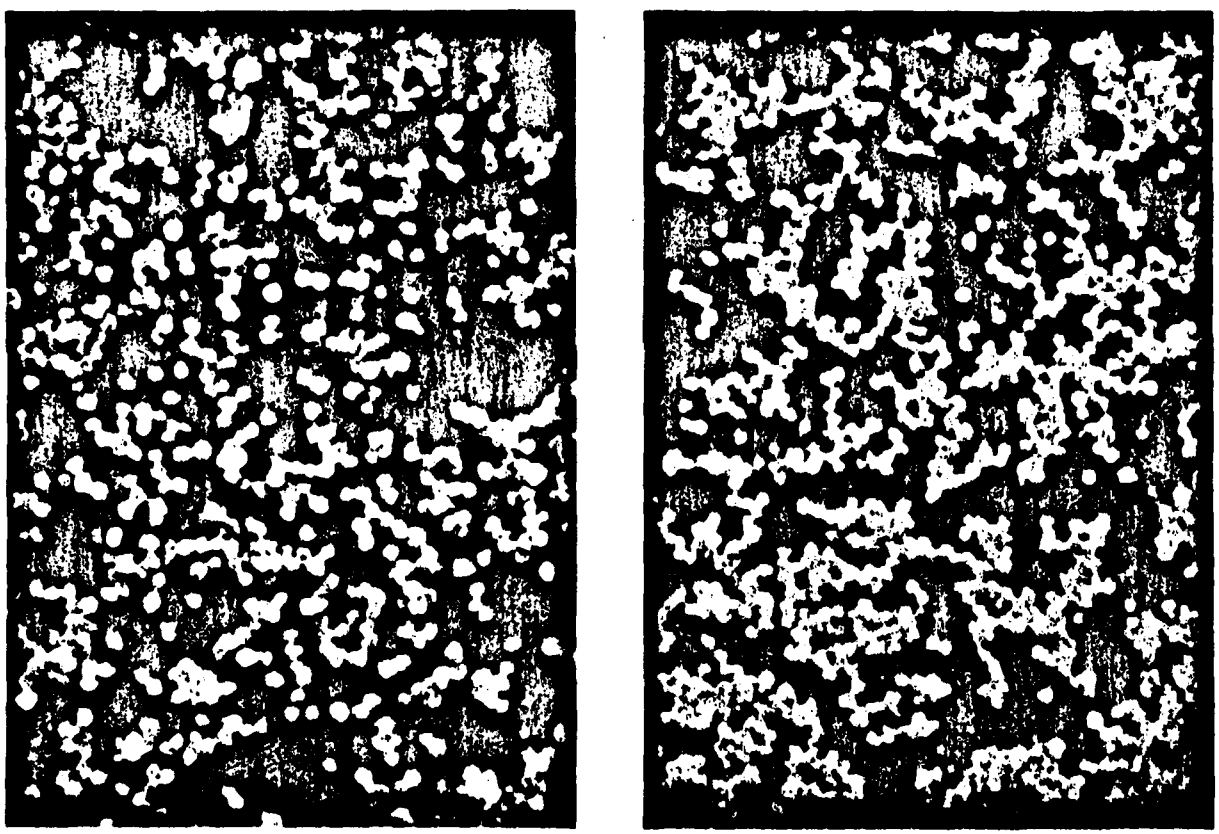


figure 10

Bibliography of Publications and Abstracts

Graham BS, and Wetherall NT: Growth of human cell lines in BALB/c mice. Cancer Research 1990;50:5943-5946.

Wetherall NT: The development of HIV-1 p24 antigenemia in the athymic "nude" mouse. In: Animal Models in Aids. Schellekens H and Horzinek MC (eds.). Elsevier; Amsterdam, 1990, pp 291-302.

Wetherall NT, Zhou J, Zhou J, and Montefiori DM: The xenotransplanted nude mouse as an animal model of HIV infection. AIDS Research and Human Retroviruses, in preparation (based upon this final report).

Wetherall NT, and Eiring A: Replication of HIV-1 in nude mice. Presented before the 28th ICAAC, Los Angeles, CA Oct. 1988, p. 303, abstracts.

Wetherall NT, and Eiring A: HIV-1 antigenemia in athymic "nude" mice. Presented before the Vth International Conference on AIDS, Montreal, Canada. June 1988, W.C.P. 31, p.597, abstracts.

Graham BS, and Wetherall N: A mouse model for investigating HIV-1 replication. Presented before the Vth International Conference on AIDS, Montreal, Canada. June 1988, W.C.P. 144, p. 615, abstracts.

Wetherall NT, and Eiring A: The development of HIV-1 antigenemia in the athymic "nude" mouse and the implications for the assessment of antiviral therapy *in vivo.*

Presented before the international TNO meeting on Animal Models in AIDS. Maastricht, The Netherlands, 23-26 October 1989.

Eiring A, Mclaughlin RL, and Wetherall NT: Distribution of p24 antigen in HIV-1 infected athymic mice. Presented before the 90th annual meeting of the American Society for Microbiology, Anaheim, CA, May 1990.

Wetherall NT, Zhou J, Li XQ, Burke A, and Montefiori DC: The suppression of p24 antigenemia in the HIV-1 xenotransplanted nude mouse. Presented before the 4th Int'l Conference on Antiviral research, New Orleans, LA, 21-26 April 1991. Antiviral Research (suppl.1) p. 129.

Personnel receiving pay from this contract support

Neal T. Wetherall, Ph.D., Principal Investigator and Assistant Professor of Pathology
(1 January 1988-29 June 1991) 45% effort

W. M. Mitchell, M.D., Ph.D., Professor of Pathology
10% effort (1 Jan 1988-28 Feb 1990)

David C. Montefiori, Ph.D., Assistant Professor of Pathology
20% effort (1 Jan 1988-28 Feb 1990)

Terrence T. Casey, M.D., Assistant Professor of Pathology
10% effort (1 Jan 1988-28 Feb 1990)

Shirley S. Schuffman, B.A., Senior Associate in Pathology 50% effort
(1 Jan 1988-28 Feb 1990)

Andrea F. Eiring, B.S., Research Assistant/Technical Supervisor III
(1 Jan 1988-9 March 1990) 100% effort

Patricia Commers, B.S., Research Assistant III
(4 Feb 1988-20 March 1989) 100% effort

Elizabeth T. Phillips, B.S., Research Assistant I
(4 Jan 1989-11 Aug 1989) 100% effort

Lori A. Crane, Research Assistant I
(24 April 1989-30 March 1990) 100% effort

Russell L. McLaughlin, B.S., Vanderbilt Medical Student I/II
(June - Oct 1989)

Heather L. Anthony, B.S., Research Assistant II
(28 Aug 1989-20 Feb 1990) 100% effort

Otho (Sonny) Armstrong, B.S., Research Assistant III
(16 April 1990-6 April 1991) 100% effort

Ada Marcelina Moreno, High School Minority Student Aid
(Sept 1990-29 June 1991)

NOTE: no graduate degrees have resulted from this contract support.

APPENDIX

CONTRACT NO.: DAMD17-88-C-8071

"ADAPTATION AND STUDY OF AIDS VIRUSES

IN ANIMAL AND CELL CULTURE SYSTEMS"

Neal T. Wetherall, Ph.D.

CONTENTS:

publication & abstract reprints

28th ICAAC, Los Angeles, Calif.

Official Abstract Form

Instructions: Complete this form and submit it for receipt by 29 April 1988. *Only this original form is acceptable (no photocopies).* Additional forms are available from the ASM Meetings Department. Refer to the sample abstract (page vi) for style. Type the title (initial capitals only) first; then list all authors (all capital letters), with an asterisk for the person delivering the paper; and then list institutions and short addresses (do not give departments, divisions, buildings, etc.). See "Preparation of Abstracts" for additional required enclosures. *Note: Any poorly prepared abstract unsuitable for direct reproduction will be retyped, and the author will be charged $20.*

Replication of HIV-1 in Nude Mice.

NEAL T. WETHERALL* and ANDREA EIRING.
Vanderbilt University School of Medicine, Nashville, TN.

Based upon the replicative cycle and unique genome of the Human Immunodeficiency Virus Type 1 (HIV-1), several therapeutic approaches for treatment of infection have been proposed. Many of these involve agents which have demonstrated activity *in vitro*, however, the efficacy of these antivirals can only be assessed through the use of a small animal model which propagates the HIV-1 *in vivo*. One such model of great potential is the utilization of the nude mouse xenotransplanted with a HIV-1 permissive human cell line. Nude mice were exposed to ^{137}Cs irradiation and inoculated with .5 or 1 X 107 human CD4+ lymphoma cells. When a palpable mass was detected, an additional inoculum of HIV-1 infected cells was delivered proximal to the mass. Ten days later, the animals were sacrificed and the tumors were harvested. Non-HIV-1 infected animals were used as controls. Southern blots of the extracted DNA were hybridized with a Bam HI-SSt 1 3'-orf fragment of pBH10R3, a DNA probe of genomic HIV. Bands at ~ 9.3 Kb and 3.8 Kb were detected. Northern blots of total RNA similarly probed detected ~ 9.3 Kb HIV genomic, 4.3 Kb env, and 1.0 Kb tat-III HIV-1 mRNA. Polyclonal sheep antibodies to viral p24 and gp 120 antigens were also detected in tumor tissue by immunohistochemical localization. These results demonstrate that the HIV-1 can proliferate in a murine system and provide the basis for development of a unique *in vivo* model for the testing of many types of HIV-1 treatment strategies.

"This work is supported by the U.S. Army Medical Research and Development Command under Contract No. DAMD 17-88-C-8071."

Category [H]

Subject category: From the list of subject categories on page v, choose the most appropriate description of the paper's content and enter the letter in the box above. *Example:* "Comparison of Methods for the Serodiagnosis of Herpes Simplex Virus Infections" should be classified as Category G.

Complete the following:

1. ☐ This material will not have been published or presented at a scientific meeting before 24 October 1988. (This includes international meetings and congresses.)
2. Full name and professional mailing address of the author who will present the paper.

Neal T. Wetherall, Ph.D
Department of Pathology C-3321 MCN Vanderbilt University
School of Medicine
Nashville, Tennessee 37232
Telephone (615) 322-2102

3. Complete checklist on opposite page before submitting abstract.

FORMULAIRE DE RÉSUMÉ

V International Conference on AIDS
Montreal, June 4 - 9, 1989

Vᵉ Conférence internationale sur le SIDA
Montréal, 4 au 9 juin 1989

Réservé au Secrétariat :
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HIV-1 ANTIGENEMIA IN ATHYMIC "NUDE" MICE

Wetherall, Neal T. and Andrea Eiring.

Department Of Pathology, Vanderbilt University Medical Center, Nashville, TN., U.S.A. 37232-2561.

Objective. Several small animal models of HIV infection have been proposed. We have developed a simple means to support the replication of HIV-1 which can produce large populations of infected mice for therapy screening and host-virus interactions.

Methods. Nude mice (3-4 wks old) were exposed to 600 R's of ^{137}Cs irradiation and inoculated with 1×10^7 HIV-1 infected CEM cells. The animals were followed daily for weight gain and tumor progression. Groups of 6 animals were exsangiunated at 8 intervals over a 9 week period and necropsies were performed.

Results. Plasma p24 antigen was detected at day 3 and rose over 9 weeks (>2200 pg/ml). Where possible, plasma antigen was neutralized with human antisera to HIV-1. The infected mice did not exhibit any weight loss, but a highly significant difference was seen in tumor progression when compared to controls. Using immunohistochemistry, sheep raised polyclonal antibodies to viral p24 and gp120 detected HIV proteins within tumor cells and mouse splenic macrophages. Electron microscopy revealed rare intracellular lentivirus particles in the CEM cells.

Conclusion. These findings suggest that the nude mouse transplanted with a well characterized HIV permissive cell line should be useful for many immunological and antiviral studies.

"This work is supported by the U.S. Army Medical Research and Development Command Under Contract No. DAMD 17-88-C-8071."

Dactylographier entre les lignes bleues : titre, noms des auteurs, affiliations, ville, province/état, pays et résumé. Souligner le nom de l'auteur-présentateur de la communication. (Se référer à l'exemple de résumé.)

1. Langue de présentation
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   Anglais [E]

2. Préférence
   Présentation orale
   Présentation d'affiche [O]

LES RÉSUMÉS REÇUS APRÈS LE 1er FÉVRIER 1989 NE POURRONT ÊTRE RETENUS

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Sous-catégorie ANIMAL MODELS Code C.1

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Kenness Canada Inc.
1010, rue Ste-Catherine Ouest, bureau 628
Montréal (Québec)
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Neal T. Wetherall, Ph.D.
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Montreal, June 4 - 9, 1989

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Montréal, 4 au 9 juin 1989

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Secretariat use only:
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Date Rcvd.
Reg. No.

A MOUSE MODEL FOR INVESTIGATING HIV-1 REPLICATION

Graham, Barney and Wetherall, N. Vanderbilt University School of Medicine, Nashville, TN, USA.

Objective. There is great need for a small animal model of HIV-1 infection. We have developed a system in mice to evaluate the effects of chemotherapy and passive antibody on HIV-1 replication.
Methods. An immunomodulation regimen was devised to allow the growth of human CD4+ cell lines CEM (lymphocytic) and U937 (monocytic) in BALB/c mice. Female, pathogen free, retired breeders underwent sublethal irradiation and depletion of L3T4+ lymphocytes utilizing the monoclonal antibody GK1.5. Mice were then inoculated with 2 X 10^7 CEM cells or U937 cells subcutaneously.
Results. By day 8 a subcutaneous plaque could be felt and by day 14 visible tumors were present. Both cell lines formed tumors that grew exponentially. If maintenance GK1.5 was administered tumor growth continued, but without maintenance tumor growth plateaued during week 3 and then regressed. Subcutaneous tumors were infected by inoculation with CEM cells chronically infected with HTLV-IIIB which resulted in HIV-1 antigenemia in mice with both CEM and U937 tumors. Mice remained active and appeared healthy throughout the experiment.
Conclusions. When standardized this system should provide an inexpensive approach to assess anti-HIV therapeutic and prophylactic agents in vivo. The approach could also be adapted to other viral systems in which small animal models are unavailable, and has potential application in many other fields of biomedical investigation.

This work is supported by the U.S. Army Medical Research and Development Command under contract No. DAMD17-88-C-8071.

Type within the blue lines: Title, Authors' names, Affiliations, City, Province/State, Country and Abstract. Underline name of presenting author. (See instructions on Sample Abstract.)

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2. Preference: Oral presentation / Poster presentation [O]

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Canada H3B 1G7

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Print name: Barney S. Graham, M.D.

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3. Do not fold the abstract.
4. An electric typewriter is preferred (no matrix printers!).
5. Please use a *black* carbon ribbon.
6. Please use single or 1½ spacing.

Original plus 2 copies to:
Animal Models in AIDS
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DEADLINE: 1st June 1989

EXAMPLE

TITLE (in capitals)

THE DEVELOPMENT OF HIV-1 p24 ANTIGENEMIA IN THE ATHYMIC "NUDE" MOUSE AND THE IMPLICATIONS FOR THE ASSESSMENT OF ANTIVIRAL THERAPY *IN VIVO*.

AUTHOR(S) (in capitals)

NEAL T. WETHERALL and ANDREA EIRING, Dept. of Pathology, Vanderbilt University School of Medicine, Nashville, TN. U.S.A. 37232-2561

Address

Abstract

Replication of human immunodeficiency virus within the mouse has been reported using diverse approaches. To develop these different systems into useful models of HIV disease, current knowledge of the unique pathogenesis of this virus must be incorporated into the design. We have produced a dynamic antigenemic state in irradiated athymic mice through the heterotransplantation of HIV-1 infected cells. In a study of matched groups of 6 mice each (total of 48 infected and 6 control animals) over a 9 week time period, the average antigen level of each group sacrificed at various time points was determined. Antigen was detectable at 3 days after inoculation (28 pg/ml), spiked at 5 days (514 pg/ml), rose steadily from 7 to 42 days (90-2181 pg/ml), then dropped off at day 63 (1269 pg/ml). Concurrent with the fall in plasma antigen levels heterotransplant growth in the infected animals increased and entered the typical exponential growth phase seen in uninfected transplants. In our studies to date, we have used human T-cell leukemia cell line, CCRF-CEM and the HTLV III_B strain of HIV-1. In preparation for future studies using other strains of HIV-1, we have transplanted and determined *in vivo* growth patterns for seven other HIV permissive cell lines: 2 T-cell, 2 monocytic leukemias, 2 carcinomas, and 1 promyelo(mono)cytic leukemia. Our system is also being extended to include bg/xid/nu mice. Strategies for therapeutic and prophylactic studies have been developed. This work is supported by the U.S. Army Medical Research and Development Command under Contract No. DAMD 17-88-C-8071.

1990 ASM Annual Meeting

Anaheim, Calif. 13–17 May 1990
Official Abstract Form

(Read all instructions before typing)

Tissue Distribution of p24 Antigen in HIV-infected Athymic Mice. EIRING, ANDREA, RUSSELL L. MCLAUGHLIN, and NEAL T. WETHERALL* Vanderbilt University School of Medicine, Nashville, TN.

Irradiated immunedeficient (athymic) mice are capable of supporting replication of HIV. Antigen levels in plasma vary over time, peaking at 6 weeks and remaining elevated for at least 9 weeks. We investigated the levels of p24 antigen in kidney, spleen, and tumor tissue at various time points during a nine week study.

Four week old ^{137}Cesium-irradiated athymic mice were inoculated with the HIV-infected tumorigenic cell line CCRF-CEM. At 1,2,3,4,6, or 9 weeks post inoculation six animals were sacrificed and plasma and tissue levels of p24 antigen were determined in an enzyme immunoassay system. Tissues were assayed by homogenizing tissue of known weight in a detergent buffer and assaying homogenates. Antigen values were standardized to pg p24/mg total protein for all tissues.

Comparison of antigen levels indicate the tumor as the primary source of virus replication, with kidney levels lower than, but paralleling, plasma levels. Spleen levels vary in a different pattern, peaking at 4 weeks. Follicular hyperplasia was noted in 28 of 30 infected mice starting at 2 weeks post inoculation, in contrast to controls (0 of 6) and 1 week post-inoculation (1 of 6). This work further characterizes the athymic mouse model of HIV infection.

Instructions

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Indicate category designation from p. ii–iii. T-9

Category designation

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Because of the flexibility in programming afforded by poster sessions, the Program Committee will attempt to schedule *all abstracts* which (i) are considered by elected divisional officers to be of acceptable quality and (ii) conform to rules established by the Program Committee. The decision of whether an abstract is scheduled in a slide or a poster session will be made by the elected Program Committee. Approximately 75% of the abstracts will be scheduled in poster sessions. By submitting an abstract, the author agrees that the paper will be presented as scheduled.

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Abstract Form

International Society for Antiviral Research

FOURTH INTERNATIONAL CONFERENCE ON ANTIVIRAL RESEARCH

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April 21-26, 1991

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The Suppression of p24 Antigenemia in the HIV-1 Xenotransplanted Nude Mouse by Azidothymidine. **N. T. Wetherall, J. Zhou, S. Armstrong, and D. C. Montefiori. Department of Pathology, Vanderbilt University School of Medicine, Nashville, TN 37232-2561.**

Murine models of HIV-1 disease are needed for the evaluation of candidate anti-HIV agents *in vivo*. On approach towards a functional model is undergoing development in our laboratory, and the results of our early studies have recently been published. This model utilizes the "nude"mouse which is well recognized as an animal model system to assay candidate antineoplastic agents. Through the transplantation of HIV-1 challenged CCRF-CEM cells into irradiated nude mice, we have demonstrated that HIV-1 p24 gag antigenemia is produced and maintained for a nine week period. To investigate whether AZT has any effect upon serum p24 levels, we administered varying does of AZT prophylactically (.25 to 2.0 mg/day/mouse p. o.) to groups of nude mice bearing human CEM cell transplants. The animals were challenged with 10^5 TCID50 units of HIV by injection of the 0.1 ml inoculum directly into the palpable mass. After 3-4 weeks of treatment the mice were sacrificed. Serum p24 levels and excised CEM cell HIV antigen expression (by IFA) were measured. The levels of these HIV indicators were significantly reduced in the AZT treated animals. To assay for other AZT effects in the nude mice, levels of AZT as high as 8 mg/day/mouse did not exhibit any mortality, morbidity, or antineoplastic effects over a 35 day period, suggesting that the inhibition of HIV is due solely to the presence of AZT. Our results indicate that this model of HIV disease can potentially be used to evaluate anti-HIV agents *in vivo* This work was supported by USAMRDC Contract No. DAMD [illegible]-88-C-8071.

Presenter's Phone No: (615) 322-2102 Fax No: (615) 343-7023

Preference: ____ Oral ____ Poster __XX__ Either

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Mail to:

Earl R. Kern, Ph.D.
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Department of Pediatrics
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Eds. H. Schellekens and M.C. Horzinek
Animal Models in AIDS

29

The development of HIV-1 p24 antigenemia in the athymic "nude" mouse

NEAL T. WETHERALL

Department of Pathology, Vanderbilt University School of Medicine, Nashville, TN 37232-2561, U.S.A.

Introduction

Recent developments toward defining animal models of human immunodeficiency virus (HIV) disease or acquired immunodeficiency syndrome (AIDS) include the successful infection of mice with the etiological agent, HIV-1. Leonard et al. (1988) have produced transgenic mice incorporating full length HIV-1 proviruses which produce offspring with infectious HIV. Namikawa et al. (1988) have reported on severe combined immunodeficiency (SCID) mice transplanted with human fetal tissue which produce infectious HIV-1. Another approach involving SCID mice, reported by Mosier et al. (1988), in which human peripheral blood lymphocytes are transplanted to reconstitute the immune system of the mice, can also support HIV-1 replication (Mosier et al., 1989).

The athymic or "nude" mouse has had utility for the study of several human infectious diseases of bacterial and parasitic (Armstrong and Walzer, 1978), mycobacterial (Colson and Kohsaka, 1982) or viral origin (Iwaski, 1978). In addition, through the process of "viral xenogenization" (Kobayashi, 1989) infectious human RNA (mumps) virus has successfully been recovered from nude mice (Reid et al., 1979). Using a similar approach, we now report the establishment of a persistent HIV-1 antigenemia in irradiated athymic mice through the xenotransplantation of virus in infected human CD4 + receptor positive (CEM) lymphoma cells. Many different human cell lines of various origins have been successfully infected with HIV-1 (Weiss, 1985). In preparation for future studies, we have transplanted and determined in vivo growth patterns for six other cell lines which may also be capable of producing a similar antigenemic state.

Materials and methods

Cell lines and virus cultures

All cells of human T-cell (CCRF-CEM or CEM, Jurkat, Molt-4), monocytic (U937, THP-1), or promyelo(mono)cytic (HL-60) origin were acquired from the American Type Culture Collection, Rockville, MD, and maintained in RPMI 1640 medium with 15–20% fetal calf serum. HeLa T4 + epithelial cells were acquired from the NIH AIDS Research and Reference Reagent Program and maintained in DMEM medium with 10% fetal calf serum. The cells were propagated at 37°C in a humid 5% CO_2 atmosphere. All of these cell lines are capable of producing infectious HIV-1 in vitro (Chesebro and Wehrley, 1988). The $TCID_{50}$ values of the human T-lymphocyte leukemia virus type III (HTLV-III) strain of HIV-1 were determined by endpoint titration of viral culture supernatants on MT-2 cells (Montefiori et al., 1988), and were standardized to produce 10^6 infectious units/ml.

Mice and cell transplantation

Athymic 3–4 week old (nu/nu) nude mice were purchased from Harlan Sprague Dawley, Inc. and were housed, maintained and inoculated as previously described (Johnson et al., 1989). All mice were exposed to 609 ($\pm$ 15) R at the body surface using a ^{137}Cesium source 24 h prior to cell transplantation. Additional precautions as outlined for biosafety level 3 were adhered to (CDC, 1988). To avoid the use of HIV-1 infected needles, a 22 gauge teflon catheter (FLASH-CATH, Travenol Labs, Inc., Deerfield, IL, U.S.A.) was inserted at the inoculation site subcutaneously, and the syringe containing 0.2 ml of CEM/HIV-1 cell suspension in serum-free RPMI 1640 medium was luer locked onto the catheter prior to injection. The animals were monitored 5 days a week for body weight and tumor presence or progression. The tumor volume was calculated from measurements in two dimensions using the formula for a prolate ellipsoid, $\pi/6$ LW^2 (Skarlin et al., 1988). Significance of differences between groups was evaluated by the unpaired Student t-test. Probability values are specified in two tails.

DNA and RNA analysis

The nucleic acids were extracted from tumor tissue, rapidly frozen in liquid nitrogen and stored at -70°C until use for DNA and RNA isolation and fractionation by methods previously described (Johnson et al., 1989). The DNA and RNA blots are modifications of the procedure of Southern (1975) and Vrati et al. (1988) respectively. The pBH10R3 plasmid is available from the NIH AIDS Research and Reference Reagent Program.

HIV-1 p24 ANTIGEN ASSAYS

The HIV-1 antigen enzyme immunoassay (EIA) is manufactured by Abbott Laboratories (Chicago, IL, U.S.A.) and is utilized on both tissue culture supernatants and heparinized mouse plasma (200 μl), acquired by exsanguination. The procedure was followed without modification (Allain et al., 1986), except that 1000 units of antigen were translated to 200 pg/ml. Background levels of reactivity using undiluted mouse plasma are similar to those of human plasma. Tumor tissue was processed for immunohistochemistry by the method described by Casey et al. (1988). The sheep anti-p24 polyclonal antibodies were purchased from Accurate Chemical and Scientific Corp., Westbury, NY, U.S.A.

Results and discussion

Our initial experiments were designed to determine if the HIV-1 permissive (Dalgleish et al., 1984), tumorigenic (Fodstad et al., 1984), CD4+ receptor positive cell line CCRF-CEM, or CEM (Foley et al., 1965), could be transplanted into gamma-irradiated nude mice and provide predictable tumor incidence and progression with minimal morbidity from irradiation or tumor burden. Various cell inocula (0.5×10^7, 1.0×10^7, 2.0×10^7, and 5.0×10^7 cells) were injected subcutaneously (s.c.) into the interscapular region of irradiated and non-irradia-

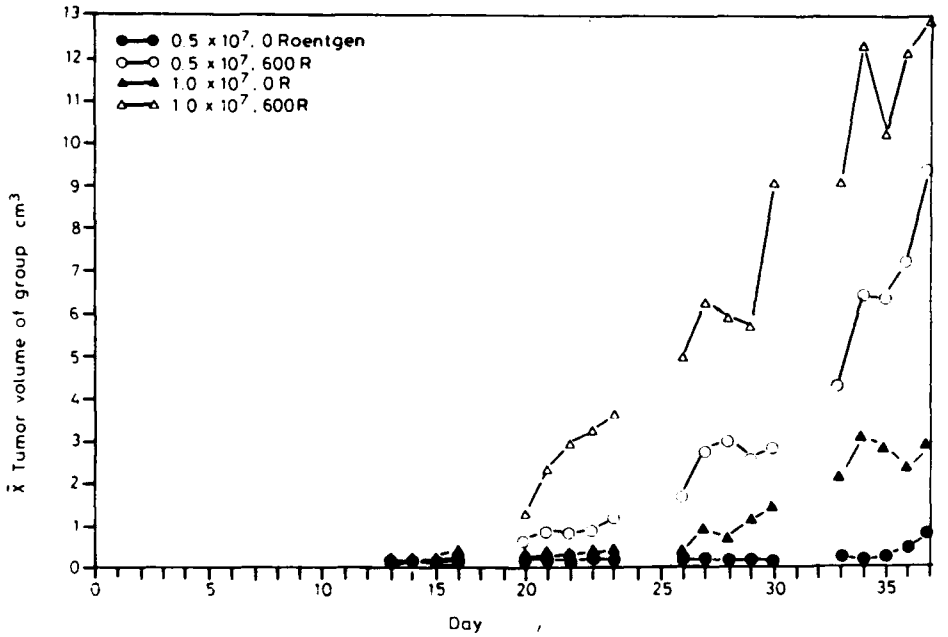

Fig. 1. Comparison of tumor volumes of CEM heterotransplants in irradiated and non-irradiated "nude" mice. Populations (n = 6) of mice were inoculated with 0.5×10^7 or 1.0×10^7 CEM cells. One group of each inoculum received 600 R ^{137}Cesium irradiation 1 day prior to inoculation.

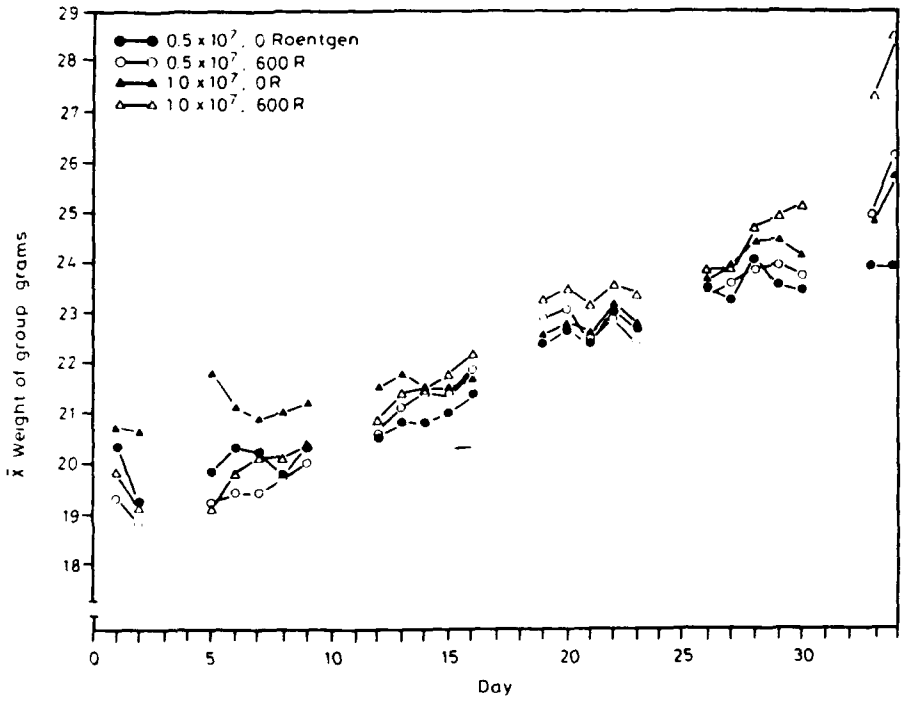

Fig. 2. Comparison of total body weight gain in irradiated and non-irradiated "nude" mice heterotransplanted with CEM cells. One group of each inoculum received 600 R ^{137}Cesium irradiation 1 day prior to inoculation.

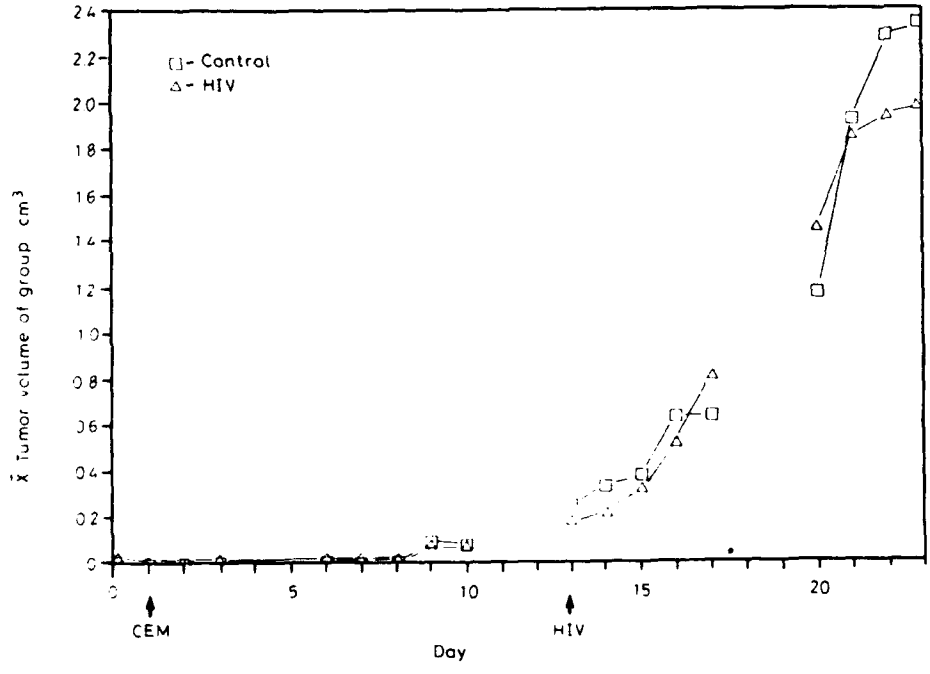

Fig. 3. Effect of HIV-1 challenge on tumor growth in "nude" mice with pre-existing CEM tumor.

ted mice. Weights and tumor volumes were monitored for 37 days. Tumors were detectable in all groups 12–14 days after inoculation. The exponential phase of tumor development was delayed by approximately 5 days in all non-irradiated groups. Regression of individual tumors occurred in 16 out of 20 non-irradiated animals. Fig. 1 shows the effects of irradiation on tumor growth in mice injected with 0.5×10^7 cells, and the optimum 1×10^7 cells. Significant differences ($P \geq 0.05$) were found between the 0.5 and 1.0×10^7 groups, and between the 1.0 and 5.0×10^7 groups. No significant differences were found between the 1.0 and

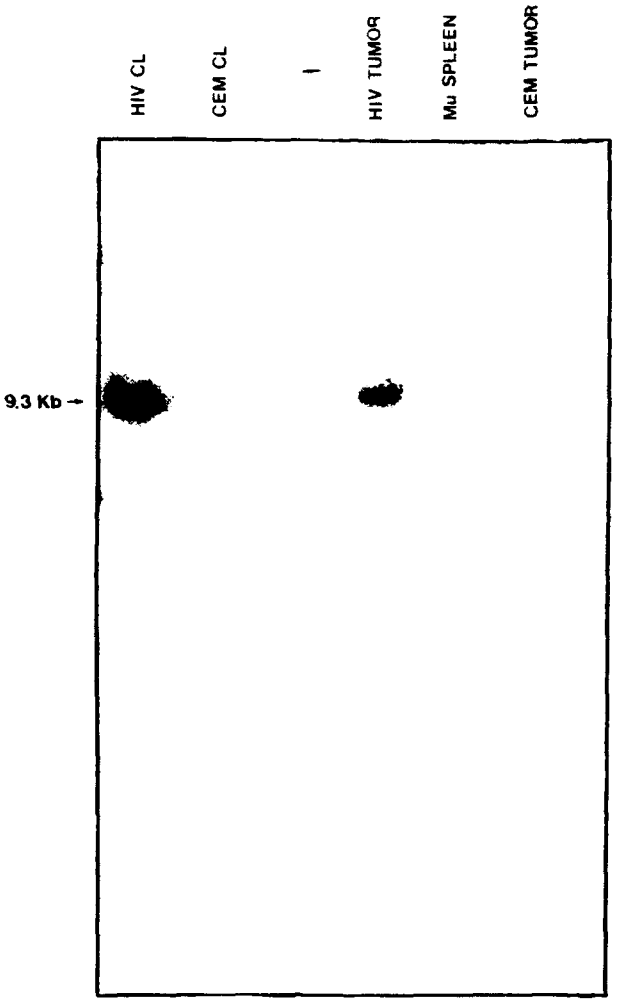

Fig. 4. Southern blot analysis of DNA extracted from HIV challenged CCRF-CEM heterotransplanted tumor. Each sample (10 μg) was digested ON at 37°C with *Sst*1 restriction endonuclease. Probe used was ^{32}P-labeled 1.1 kb HIV *nef* sequence from pBH10R3 plasmid. lane 1, CEM cell line infected with HTLV-IIIB strain of HIV-1; lane 2, uninfected CEM cell line; lane 3, tumor from infected cells; lane 4, mouse spleen; lane 5, tumor from uninfected cells.

2.0×10^7 groups, however the 1×10^7 inoculum produced consistent tumor growth without tumor necrosis. Fig. 2 shows the weight gain and the ability of the animals to thrive in these same groups of mice was similar, indicating that there were no harmful effects from the irradiation.

In order to determine if an established transplant could support the replication of HIV-1, six mice were challenged with HIV-1 chronically infected CEM cells. An inoculum was injected directly into the palpable mass created by inoculating 1×10^7 uninfected CEM cells s.c. 13 days earlier. The mice were followed for 10 days. Fig. 3 indicated that using this approach, the virus had no effect on tumor

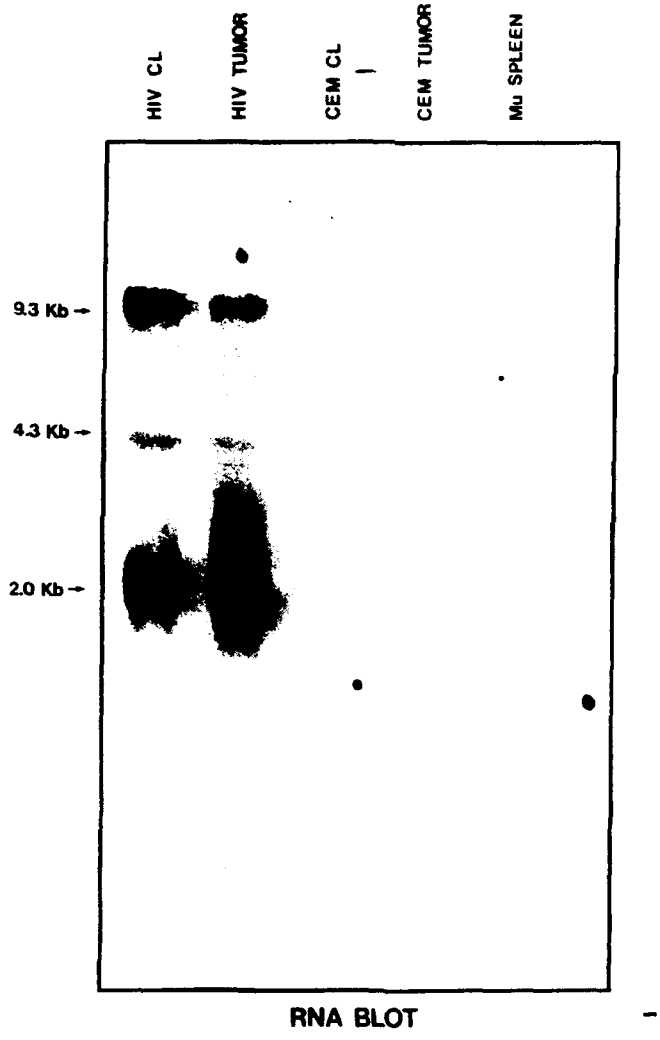

Fig. 5. Northern blot analysis of total cellular RNA from HIV challenged CCRF-CEM heterotransplanted tumor. Blot was probed with the same ^{32}P-labeled *nef* sequence used for Southern analysis. Lane 1, CEM cell line infected with HTLV-IIIB strain of HIV-1; lane 2, tumor produced in "nude" mouse with infected cells; Lane 3, CEM uninfected cell line; lane 4, tumor from uninfected cells; Lane 5, mouse spleen. All samples were 20 μg of total cellular RNA.

progression. Previous reports (Ratner et al., 1985; Wain-Hobson et al., 1985) indicate that homology exists between the HTLV-III/LAV strains of HIV-1 and the Moloney murine leukemia virus (Mo-MuLV). To determine the integrity of the 1.1 kb *nef* gene probe, a Southern blot of infected and non-infected CEM tumor and nude mouse spleen DNA was performed. The findings displayed in Fig. 4 indicate that HIV-1 proviral DNA can be detected in the tumor mass, without cross-reacting with murine cells or potentially present murine retrovirus. Viral replication, and not solely integration, was determined by performing a Northern blot (Fig. 5) on the RNA from the same tissues. The four mRNA moieties, i.e., from the 9.3 kb genomic, 4.3 kb envelope, and the approximately 2.0 kb *tat* and *nef* genes, are expressed only in the control cell line and the infected tumor tissue. The findings offered by the nucleic acid analyses strongly suggest that non-murine antibodies raised against HIV-1 proteins should be specific, and would not cross-react with other potentially present retroviral proteins.

Presence of the major core p24 *gag* protein of the HIV-1, p24 *gag*, circulating in the bloodstream, as detected by antigen capture assays, has significant clinical implications. Variations in the plasma antigen levels in HIV-1 infected individuals is accepted as an important indicator of disease progression in both hemo-

Fig. 6. Stained tissue section of HIV-1 infected CEM tumor transplanted into a "nude" mouse reacted with a sheep polyclonal antibody to p24 HIV core protein. Peroxidase conjugated secondary antibody was developed with diaminobenzidine as substrate.

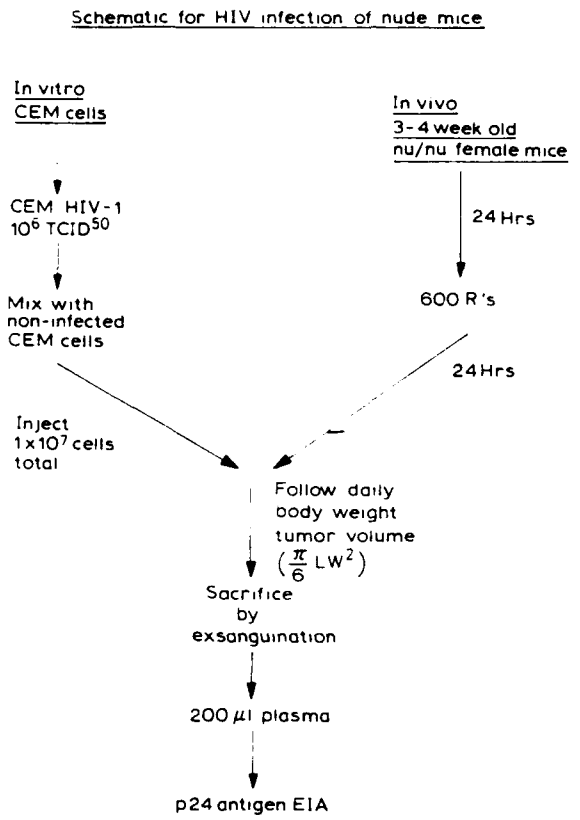

Fig. 7. Scheme for the production of HIV p24 anigenemia in "nude" mice.

philiac (Allain et al., 1986) and homosexual (Lange et al., 1986) populations. In addition, a suppression in plasma levels of p24 antigen is associated with a positive therapeutic response to candidate antiviral agents (Chaisson et al., 1986). Therefore, we determined the presence of p24 antigen in tumor tissue by immunohistochemistry and in plasma by EIA with antibodies raised in sheep and rabbits respectively. The tumors from the animals infected in the initial experiments were stained using an immunoperoxidase method and revealed numerous cells with cytoplasmic positivity for p24 antigen (Fig. 6). Control tissue was negative and without background (not shown).

An approach was developed to determine if p24 antigen could readily be detected in mouse plasma, and is depicted in Fig. 7. This scheme was followed by preparing irradiated 4 week old nude mice, and injecting the optimum 1×10^7 inoculum of CEM cells, which were first mixed in a ratio of 90% uninfected and 10% HIV-1 infected cells. We determined the course of antigenemia over a 9 week period in matched groups of six HIV-1 infected nude mice (total of 48 infected and six control animals). At each test point, one group of six mice was exsanguinated and killed by cardiac puncture and plasma p24 antigen levels were determined at various time points. Fig. 8 shows the average antigen level of the group at each time point. Antigen was detectable at 3 days after inoculation (28

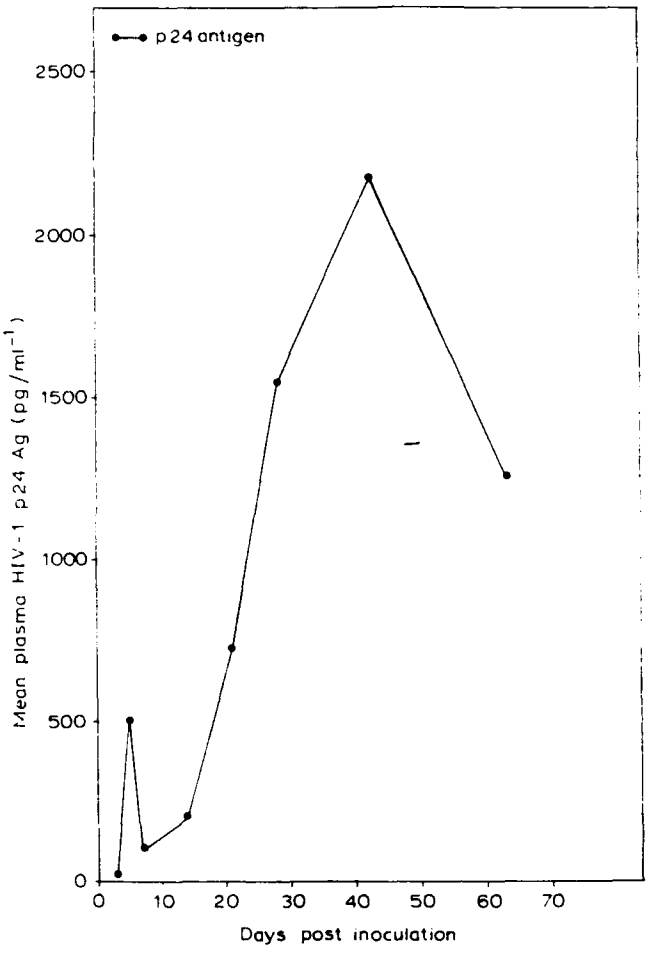

Fig. 8. Average antigen plasma levels of mice heterotransplanted wih HIV-infected CCRF-CEM cells. Each point represents the average of six animals.

pg/ml), spiked at 5 days (514 pg/ml), rose steadily from 7 to 42 days (90–2181 pg/ml), then dropped off at day 63 (1269 pg/ml). It is not clear at this time what is responsible for this dynamic antigenemic state, but it could be due to either the mouse immune system (Holub, 1989), the viral replicative cycle (Gallo et al., 1984), or CD4 + receptor regulation by the CEM cells (Salmon et al., 1988), however the antigen curve indicates a strong similarity to in vitro HIV-1 replication (Montefiori and Mitchell, 1987). No mortality or disease symptoms from HIV-1 infection were observed in any of these mice.

The pathogenesis of HIV disease is complex and the course of the disease may be influenced by the type of cells which are infected with the virus (Levy, 1989). Therefore, using 1×10^7 cells as an inoculum, we determined if other HIV permissive cell lines could produce successful heterotransplants in the irradiated nude mouse system. Seven cell lines (including CEM) of four different cell lineages were injected into populations of six mice each, and the tumor progres-

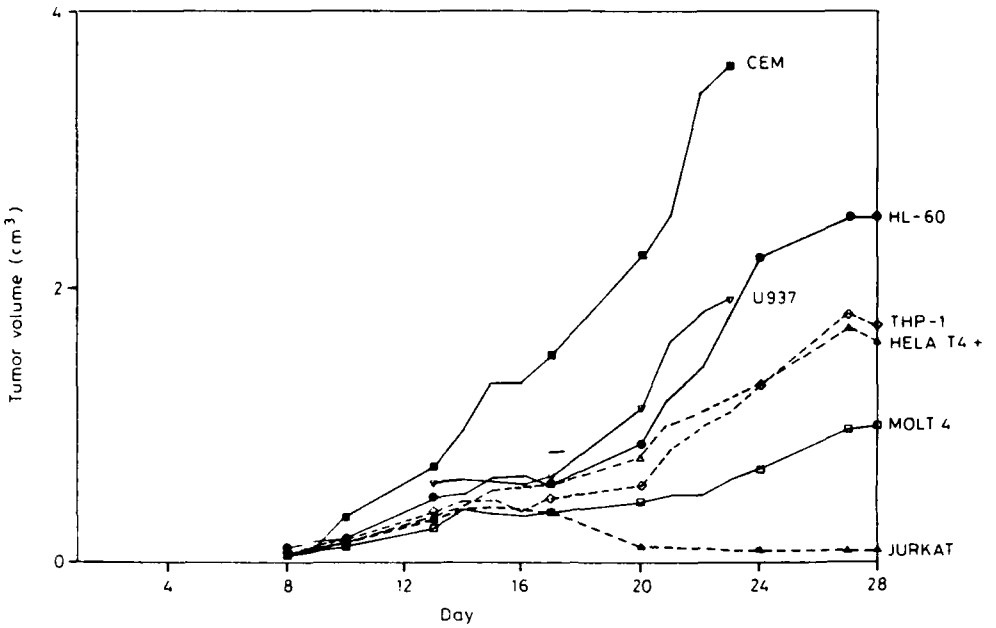

Fig. 9. Tumor progression of several HIV permissive cell lines heterotransplanted into "nude" mice. All mice received 600 R ^{137}Cesium irradiation and 1×10^7 cells.

sion is shown in Fig. 9. All of the cell lines developed palpable masses by day 8 and progressively grew except for the CD4 + positive lymphoma derived Jurkat, which regressed starting at day 17. The T-cell derived Molt-4 did progress in tumor volume, however not in a similar manner to the CEM cells. These findings indicate that differences exist among these cells which may influence the antigenemic state when infected cells are transplanted. Although several titers of inocula need to be assayed, the other cells of hematopoietic or epithelial origin which were utilized in this study all developed progressively expanding masses in the mice with a 1×10^7 cell inoculum, and should prove to have utility to study the role of HIV infection and therapy in cells of differing phenotypes.

The results reported here demonstrate a system for the infection of nude mice with HIV-1 which produces a dynamic measurable antigenemic state. This clinically relevant parameter provides the means to investigate several aspects of HIV infection. We anticipate this model system will prove useful to assay and study candidate anti-HIV therapies as well as offer insight into other aspects of HIV disease such as host immunomodulation, passive immunotherapy, host/HIV strain isolate interaction, and in vivo virus mutation.

Acknowledgements

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In conducting research using animals, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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Growth of Human Cell Lines in BALB/c Mice[1]

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ABSTRACT

A method of immunodepletion that allows the growth of xenogeneic cell lines in mice is described. Treatment of adult BALB/c mice with sublethal irradiation (400 total body rads) and CD4⁺ lymphocyte depletion renders them tolerant to the growth of human lymphocytic (CEM), monocytic (U937), and epithelial (HEp-2) cell lines. The system has also been used to generate ascites from rat hybridomas. The methodology is technically easy, inexpensive, and expedient and should have multiple uses in biomedical research including mouse modeling of human viral infections and investigation of transplant rejection.

INTRODUCTION

The induction of specific tolerance to foreign cells has been a major goal of immunology because of its relevance to tissue allografts, organ transplantation, and control of allergic and autoimmune diseases. *In vivo* depletion of specific T-cell subsets is one approach to achieving such tolerance and has been explored in allogeneic marrow and skin grafts (1–3) and in eliminating the antibody response to soluble antigen (4–6).

We have developed a protocol combining sublethal irradiation and *in vivo* depletion of CD4⁺ lymphocytes that allows human cell lines to grow in a standard inbred strain of mice. The ability to grow human cell lines in mice has several biomedical research applications. The potential for *in vivo* investigation of human viral pathogens that lack small animal models is apparent, and particularly relevant to HIV-1. The ability to grow human CD4-positive lymphocytic and monocytic cell lines in mice may provide a relatively easy and inexpensive method for investigating HIV replication *in vivo*.

MATERIALS AND METHODS

Animals. Pathogen-free female retired breeder BALB/c mice were purchased from Charles River Laboratories, Raleigh, NC. They were housed in a Duo-Flo laminar flow chamber (Lab Products, Inc., Maywood, NJ); sterile cages, bedding, food, and water were used. At least 4 mice were included in each experimental group. In conducting research using animals, the investigators adhered to the standards described in Ref. 7.

Cells. CEM (ATCC[2] CCL-119) is a human CD4⁺ lymphocytic cell line derived from a patient with acute lymphoblastic leukemia. U937 (ATCC CRL-1593) is a human CD4⁺ monocytic cell line derived from a patient with diffuse histiocytic lymphoma, and HEp-2 (ATCC CCL-23) is a human epithelial cell line derived from a man with laryngeal carcinoma. CEM, U937, and HEp-2 cell lines were obtained from the American Type Culture Collection. CEM and U937 were grown in RPMI 1640 Medium containing 20% heat-inactivated fetal bovine serum without antibiotics, and HEp-2 were grown in Eagle's minimal essential medium containing 10% fetal bovine serum with penicillin (200 units/ml), and gentamicin (50 μg/ml) and amphotericin (1 μg/ml). All cell lines were monitored for the presence of *Mycoplasma* by rRNA hybridization (Gen-Probe, San Diego, CA) and were found to be negative. The CEM and U937 cells were routinely monitored for HIV contamination by indirect fluorescent antibody labeling and reverse transcriptase assays. Results from testing of these lines were negative.

Antibody. GK1.5 is a rat IgG2b monoclonal antibody specific for L3T4, the CD4 determinant for mouse lymphocytes (8). The hybridoma producing monoclonal antibody GK1.5 was a gift from Steven Martin, University of Tennessee at Knoxville. Ascites was produced in BALB/c-*nu/nu* mice exposed to 450 TBR and primed with pristane. After clarification by centrifugation at 2000 × *g* for 20 min, the ascites was pooled. Total protein and albumin concentrations were quantitated using a Multistat III Microcentrifugal Analyzer (Instrumentation Laboratory, Lexington, MA). Serum protein electrophoresis was performed with a Titan Gel high-resolution REP SP-30 kit (Helena Laboratories, Beaumont, TX) and the γ-globulin fraction was determined by densitometry with an Electrophoresis Data Center (Helena Laboratories). Ascites was diluted in phosphate-buffered saline to 500 μg of monoclonal antibody per ml prior to injection.

Immunodepletion and Injection of Mice. Mice were given injections of 100 μg of GK1.5 i.p. on 3 successive days and received 450 TBR from a Mark I cesium-137 irradiator on day 2. Mice were given s.c. injections with 2×10^7 cells in 0.3 ml of serum-free media on day 3 following the third injection of GK1.5. Maintenance injections of GK1.5 (250 μg) were given weekly. Verification that the appropriate lymphocyte subset was depleted was performed by FACS analysis of heparinized whole blood using fluorescein isothiocyanate or phycoerythrin-conjugated rat anti-mouse antibody to CD8 or CD4, respectively (Becton Dickinson, Mountainview, CA). Labeled cells were prepared by the whole blood lysis method and analyzed on a Coulter Epic 753 FACS.

RESULTS

Growth of Human Lymphocytic Cell Line in Mice. Immunodepleted mice given injections of CEM developed palpable s.c. plaques between 8 and 14 days post-injection. The tumors grew exponentially as long as GK1.5 treatments were maintained. The percentage of CD4-positive lymphocytes in peripheral blood of mice receiving maintenance GK1.5 injections were 2.1% ± 1.9%. Without GK1.5 maintenance injections, tumors began regressing during the third week (Fig. 1). FACS analysis of pooled peripheral blood on day 16 after the last GK1.5 treatment showed 13% CD4-positive cells. CEM tumors reached a large size after 5 weeks of growth without sign of illness in mice and without signs of rejection in the tumor (Figs. 2 and 3). Tumors did not grow in mice subjected to GK1.5 treatment or irradiation alone.

Growth Characteristics of Human Monocytic and Epithelial Cell Lines in Mice. In addition to the lymphocytic cell line CEM, monocytic and epithelial cell lines (U937 and HEp-2, respectively) were investigated in mice immunodepleted using the same regimen. U937 grew exponentially to large size leading to cutaneous ulceration, after which its growth plateaued (Figs. 3 and 4). All mice given injections of HEp-2 developed tumors that were palpable by day 8 to 10, but after growing to a size of 8 to 12 mm diameter, no further growth occurred (Fig. 5). The

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[2] The abbreviations used are: ATCC, American Type Culture Collection; HIV, human immunodeficiency virus; TBR, total body rads; FACS, fluorescent-activated cell sorter.

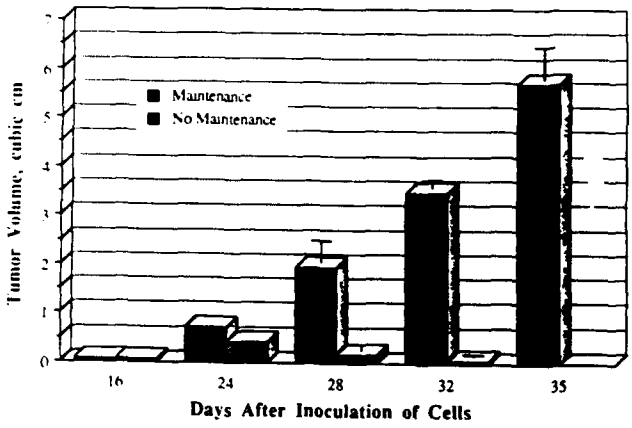

Fig. 1. Illustration of how maintenance of CD4+ lymphocyte depletion by weekly i.p. injections of GK1.5 affects tumor growth. Tumors in mice receiving maintenance injections of GK1.5 (■, 4 mice) continue to grow, whereas tumors in mice that receive no maintenance injections of GK1.5 (□, 3 mice) begin receding after 3 weeks. Tumor volume was determined by the formula, V = length × width² × $\pi/6$, for a prolate ellipsoid. *Bars*, SE.

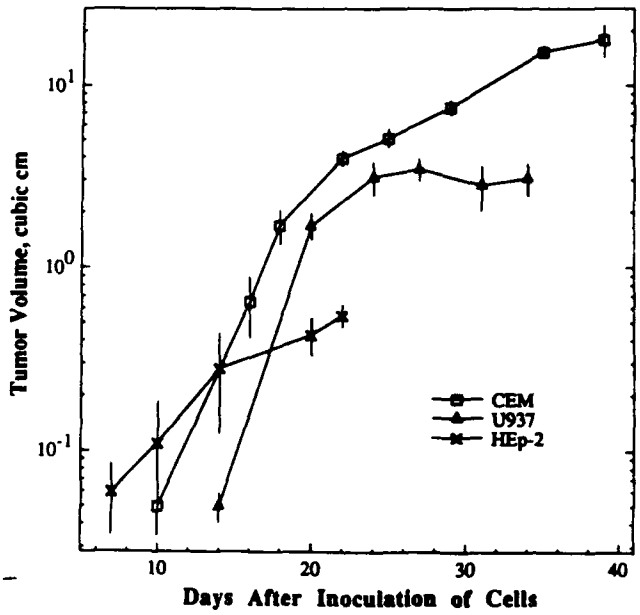

Fig. 3. Kinetics of CEM (4 mice), U937 (4 mice), and HEp-2 (3 mice) cell growth in immunodepleted mice. Tumor volume was determined by the formula, V = length × width² × $\pi/6$, for a prolate ellipsoid. *Bars*, SE.

tumor size remained constant for 10 days prior to harvest (Fig. 3). Both U937 and HEp-2 tumors appeared healthy and free of rejection at the time of harvest (Figs. 4 and 5). Combining the results of all experiments using the basic GK1.5 depletion/irradiation preparation, the total numbers of tumors per attempted mouse were 15 of 17 for CEM, 18 of 18 for U937, and 3 of 4 for HEp-2.

Immunodeterminants of Human Cell Growth in Mice. Mice receiving either irradiation or GK1.5 treatment alone did not allow growth of CEM cells, suggesting that effector mechanisms other than CD4-positive lymphocytes were involved in the rejection phenomenon. To determine the effect of GK1.5 and irradiation on memory cell capacity to mediate rejection, these same mice were allowed to recover for 1 month. Both groups were then subjected to 450 TBR and GK1.5 treatment and given 2×10^7 CEM cells s.c. (Table 1, experiment 1). No tumors developed, suggesting that a CD4-negative, relatively radioresistant lymphocyte population was responsible for the rejection phenomenon or that a small number of residual CD4-positive cells is sufficient to mediate the rejection response. Groups of control mice in each phase of the experiment developed tumors. Peripheral blood CD4-positive lymphocytes were undetectable in GK1.5-treated groups, whereas untreated mice or mice treated with irradiation alone had 27 ± 8% CD4-positive peripheral blood lymphocytes.

Another experiment was designed to look at the role of CD8-positive lymphocytes in rejection and the potential for heterologous cell priming of the immunological effectors determining rejection. Mice were divided into 4 groups (Table 1, experiment 2). Two groups were immunized with 10^7 CEM and 1 group with 10^7 HEp-2 i.m.; the other group received nothing. Four

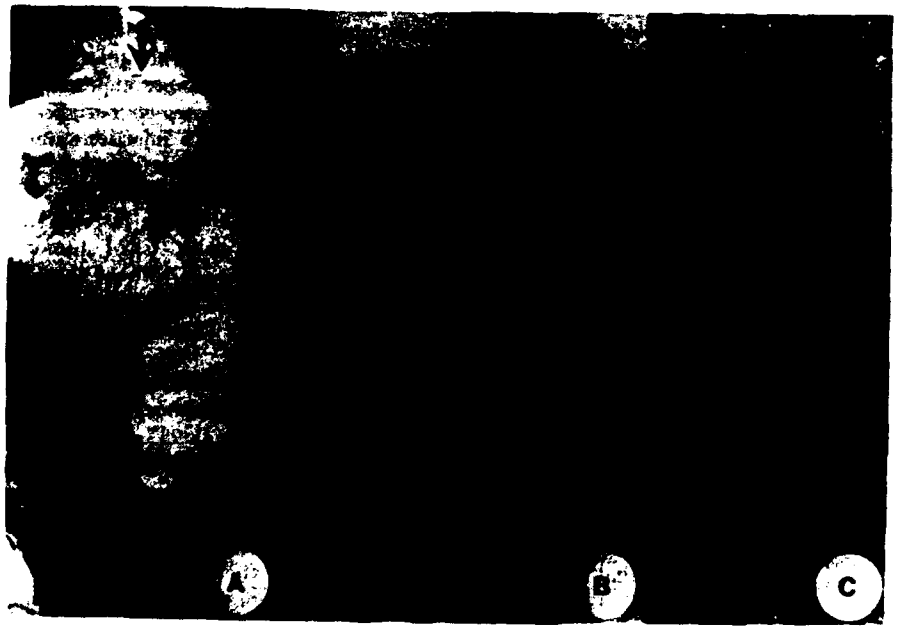

Fig. 2. CEM growth in mouse prior to harvest (*A*), *in situ* (*B*), and histologically (*C*). H & E, × 250). Note lack of inflammation and multiple mitotic figures in *C*. *Arrows*, perimeter of tumor.

Fig. 4. U937 growth in a mouse histologically (*A*) and prior to harvest (*B*). Skin overlying the tumor was ulcerated, but the tumor itself did not appear necrotic and had no histological evidence of inflammation.

Table 1 *Preliminary evaluation of the immunodeterminants of rejection*

No. of mice	Initial preparation of mice[a]	No. of tumors after challenge with CEM cells s.c.	Secondary preparation of mice[a]	No. of tumors after challenge with CEM cells s.c.
Experiment 1				
4	GK1.5	0/4	GK1.5/X-ray[b]	0/4
4	X-ray	0/4	GK1.5/X-ray	0/4
4	GK1.5/X-ray	4/4		
4			GK1.5/X-ray	4/4
Experiment 2				
4			GK1.5/X-ray	2/4
4	HEp-2		GK1.5/X-ray	0/4
4	CEM		GK1.5/X-ray	0/4
4	CEM		GK1.5/2.43/X-ray	0/4

[a] GK1.5 monoclonal antibody injected i.p., HEp-2 and CEM injected s.c.
[b] X-ray, 450 TBR from a cesium source.

weeks later, all mice received 450 TBR and the GK1.5 depletion regimen. One of the CEM-immunized groups also received a similar regimen of the monoclonal antibody 2.43, which is a rat IgG2B that recognizes murine CD8. All groups were challenged with 2×10^7 CEM s.c. and were then observed for tumor development. Only the unimmunized group developed tumors, suggesting that heterologous human cell immunity is sufficient to induce rejection, and that depletion of CD8-positive cells is not sufficient to restore tumor susceptibility in immunized mice. CD8-positive lymphocytes comprised 8.2 ± 2.7% of peripheral blood lymphocytes in untreated controls or those treated with GK1.5 alone, but were undetectable (0.19 ± 0.15%) in 2.43 treated mice.

DISCUSSION

The ability to grow human cell lines in normal mice immunodepleted with readily available reagents should have many applications in biomedical research. The methods outlined are

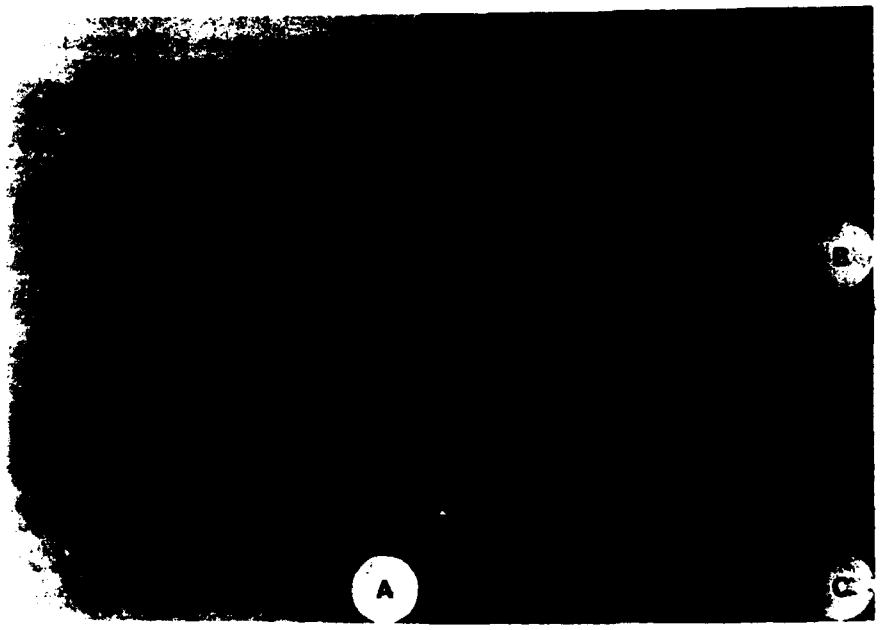

Fig. 5. HEp-2 growth in a mouse *in situ* (*A*) and histologically (*B* and *C*). Although tumor growth had plateaued, there was no evidence of necrosis. *Arrows* outline perimeter of tumor. Histologically there was evidence of cell death, but most tumor cells appeared healthy (*B*). There was no appreciable inflammatory reaction, but bands of fibrous tissue had infiltrated the tumor to cause septation (*C*).

inexpensive, quick, and technically easy, and mice remain healthy in a routine isolation facility. Our primary interest is in using this as a vehicle for investigating HIV replication *in vivo*. The system, however, might be modified for the study of other human pathogenic viruses that can be cultivated *in vitro* but lack a susceptible small animal model. Although the system is limited in its ability to answer questions concerning viral pathogenesis, it provides an *in vivo* system to assess prophylactic or therapeutic strategies designed to interfere with viral replication.

There are also potential applications of this system for investigating the cellular immunology of transplant rejection. Mice immunized with cells prior to initiating the immunodepletion regimen will not allow growth of those cells on rechallenge (data not shown). This suggests that a CD4-negative, radioresistant memory cell population, capable of rejecting foreign cells without CD4 help, exists. A similar finding has been noted in GK1.5-treated mice given skin allografts where $Lyt2^+$ (CD8) cells dominate the secondary graft response seemingly without the need of CD4 help (3). However, CD8 depletion in our system did not restore a state of tolerance to the growth of human cells after immunization with autologous cells. It is not known whether this indicates that a CD4-negative, CD8-negative cell population is responsible for rejection, or whether immunization leads to cell populations more resistant to radiation and Mab depletion, or whether relatively small numbers of residual undepleted CD4-positive or CD8-positive memory cells are capable of mediating the rejection response. The finding that immunization with HEp-2 (a human epithelial cell) primes mice for rejection of CEM (a human lymphocytic cell) suggests that nonspecific immune mechanisms were activated, or that recognition of shared human cell-associated proteins was sufficient to induce rejection.

The spectrum of uses for this immunodepletion regimen is similar to that for nude mice. Aside from cost, the advantages of this system over nude mice are that once the immediate effects of irradiation have faded, the immunologic defect is confined to the CD4-positive cell population. This leaves the animals less susceptible than nude mice to adventitious agents over the short term of 3 months, and yet completely lacking an antibody response to new antigens. The major disadvantages compared with nude mice are the need for maintenance GK1.5 injections on a weekly basis, and the extensive experience with nude mice in a number of experimental systems. One practical use of the immunodepletion regimen is production of ascites from non-murine hybridomas. Using adult BALB/c females, we have produced high-titered ascites from 7 different rat hybridomas. This approach produced more ascites, and was less expensive and easier than using nude mice. Adapting the system for the growth of hormone-responsive or hormone-sensitive cells would provide a model for studying endocrine regulatory mechanisms and secretory products *in vivo*. If the system can be adapted to growth of primary cells, the study of tumorigenesis or organogenesis might also be possible. An application of immediate clinical relevance would be to use the system for the growth of neoplastic tissue from patients, which could then be subjected to *in vivo* sensitivity testing with chemotherapeutic agents.

Having tested a number of cell lines using this immunodepletion regimen, we are encouraged by the consistent ability to grow tumors in mice from human cell lines of lymphocytic, monocytic, and epithelial cell origins. Creative use of specific immunodepletion regimens and growth of human cells in mice will be a valuable tool for many areas of biomedical research.

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